ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 301

From the Department of Pharmacology Royal Veterinary College Stockholm Sweden

SITES OF STEROID HORMONE FORMATION

AUTORADIOGRAPHIC STUDIES USING LABELLED PRECURSORS

BY

LARS ERIK APPELGREN

STOCKHOLM 1967

NOMENCLATURE

Trivial names and systemic names of the steroids referred to in this book

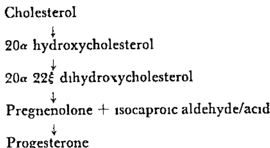
d aldosterone	11β 21 dihydroxy 3 20 dioxopregn 4 en 18 al
cholestrol	cholest 5 en 3β ol
corticosterone	11β 21 dihydroxypregn 4 ene 3 20 dione
cortisol	11β 17α 21 trihydroxypregn 4 ene 3 20 dione
cortisone	17α 21 dihydroxypregn 4 ene 3 11 20 trione
dehydroepiandrosterone	3β hydroxyandrost 5 en 17 one
16 dehydroprogesterone	pregna 4 16 diene 3 20 dione
deoxycorticosterone	21 hydroxypregn 4 ene 3 20 dione
estradiol (17β)	estra 1 3 5(10) triene 3 17β diol
estrone	3 hydroxy estra 1 3 5(10) trien 17 one
18 hydroxy 11 deoxycorticosterone	18 21 dihydroxypregn 4 ene 3 20 dione
17α hydroxypregnenolone	3β 17α dihydroxypregn 5 en 20 one
17α hydroxyprogesterone	17α hydroxypregn 4 ene 3 20 dione
pregnenolone	3β hydroxypregn 5 en 20 one
progesterone	pregn 4 ene 3 20 dione
testosterone	17β hydroxyandrost 4 en 3 one

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INTRODUCTION

Cholesterol has been established as a key precursor of the steroid hormones although it may not be an obligatory intermediate in steroid hormone bio synthesis (Heard et al 1956 Hechter 1958 Caspi 1961 Shimizu and Gut 1965) The generally accepted sequences of reactions are based on work with perfused ox adrenal glands (Hechter et al 1951 Hechter et al 1953) Later investigations have resulted in modifications of and additions to the original schemes but the early general scheme is still mainly accepted (cf Grant 1960) The following sequence may serve as an orientation of the path way for the formation of progesterone from cholesterol (cf Wettstein 1961 Grant 1962 Shimizu et al 1962 Dorfman and Sharma 1965)



The pathway from cholesterol to pregnenolone appears to be common in all steroid secreting glands (Grant 1962)

The enzymes involved at the different stages have been extensively studied (for review see Dorfman 1955 Wettstein 1961 Grant 1962 Talalay 1965) The most studied enzyme is the Δ^5 3β hydroxysteroid dehydrogenase which acts on e g pregnenolone to form progesterone The histochemical demonstra tion of this enzyme and other dehydrogenases has been extensively used by many authors to indicate the sites of steroid hormone production using dif ferent hydroxysteroids as substrates (cf Baillie et al 1966) There seem how ever to be very few histological investigations dealing with enzyme reactions in the steps between cholesterol and pregnenolone Ferguson (1966) was not able to show any Δ^5 3β hydroxysteroid dehydrogenase in the rat ovary when cholesterol was used as a substrate nor was any reaction shown in the human ovary with cholesterol as a substrate (Ferguson and Hart 1966) However Hardonk (1965) has shown the localization of a secondary alcohol dehydro genase in human steroid producing cells and suggests that this enzyme is involved in the oxidative cleavage of the cholesterol side chain

Localization of possible sites of synthesis of steroid hormones has also been made with histochemical demonstration of endogenous cholesterol e g Schultz

reaction and the bismuth trichloride method (cf Pearse 1960) Demonstration of ketosteroids has been performed by Stoward and Smith (1964) among others Although the specificity of these histochemical methods is debatable they have contributed to better knowledge of the localization of sterols

The classical method to study cholesterol by its birefringence is of value in scanning tissues but its results should be viewed with caution since a variety of unsaturated lipids and other compounds show this reaction (cf Pearse 1960)

Most studies have been made on the adrenal and reproductive glands in a variety of animal species It is generally agreed that the zona fasciculata of the adrenal is the portion richest in cholesterol and its esters (for review see Bourne 1958) and the pattern of this zone and the other adrenal zones is affected by age sex and physiological state of the animal

The testes of various animals have been shown to contain cholesterol mainly in the interstitial cells (cf Pollock 1942 McEnery and Nelson 1950 Melampy and Cavazos 1954) but in humans Montagna and Hamilton (1951) suggested that "the presumptive sites of hormones should include the interstitial fibroblast like cells and the seminiferous epithelium as well as the cells of Leydig

The ovary has been investigated by histochemical methods in a variety of experimental animals (for review see Deane and Seligman 1953) From the ... of various investigators a general conclusion may be drawn that the ... ount of cholesterol in the interstitial tissue the theca and the corpora lutea varies with species age phase in sexual cycle and pregnancy

The present autoradiographic investigation was made to obtain information about the distribution *in vivo* of some steroid ... i e cholesterol pregnenolone and progesterone A better kn... the distribution pattern of these substances and/or their metabo... of steroid hormone formation as well as in other ... contribute to the understanding of the compl... formation

The whole body autoradiographic technique ... 1958) has been used to get the gross distribution ... Detailed studies on the distribution in some ... according to a modification of a method described ... 1965) Quantitative determinations of C^{14} in ... the labelled substances have been carried out as ... radiographic investigation Some chromatograph... order to elucidate what the majority of the C^{14} ... represents in the different cases The sites of ... also studied by histochemical localization of hyd... activity in whole body ...tions from mice A preli... has been presented (Appelgren 19..)

DISTRIBUTION OF C^{14}-4-CHOLESTEROL AND C^{14}-26-CHOLESTEROL IN MICE

Although there is much information on cholesterol contents estimated both with chemical and histochemical methods in various organs of humans and different animals (for review see Cook 1958 Kritchevsky 1958) there are few *in vivo* studies of the uptake and kinetics of exogenous cholesterol in sites of steroid hormone synthesis (cf Eik Nes 1967 Samuels and Uchikawa 1967)

In the following study two differently labelled cholesterol compounds were used C^{14}-4 cholesterol and C^{14} 26 cholesterol The use of these two substances gives some indication of the chemical fate of labelled cholesterol In experiments with C^{14} 26 cholesterol (side chain labelled) the radioactivity cannot represent steroid hormone products as the labelled side chain is split off when steroid hormones are formed (Fig 2) The C^{14} 4 cholesterol (ring labelled) can be transformed to labelled steroid hormones which may be traced to target organs (Fig 1)

Whole body autoradiography was used to study the distribution of the two differently labelled cholesterol compounds with special reference to sites of steroid hormone synthesis Survival times ranging from 2 minutes to 60 days have been studied

Because of difficulties to prepare even emulsions with the very small quantities of labelled substances for intravenous injections in mice the substances were administered in oil subcutaneously or intramuscularly In order to be able to study the kinetics after an intravenous injection which allows short time distribution studies a method for injection of cholesterol dissolved in small amounts of ethanol was developed

A semiquantitative determination of the contents in various organs was made with a grey scale This grey scale was made from an isotope staircase consisting of small pieces of film that had been dipped in different concentrations of a C^{14} solution and then autoradiographed together with each whole body section (Berlin and Ullberg 1963)

To get a more precise localization of the radioactivity within some selected organs a microautoradiographic study was made according to a recently developed method (Hammarström et al 1965)

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The present autoradiographic investigation was made to obtain more detailed information about the distribution *in vivo* of some steroid hormone precursors i e cholesterol pregnenolone and progesterone A better knowledge of the distribution pattern of these substances and/or their metabolites in sites of steroid hormone formation as well as in other tissues of the body might contribute to the understanding of the complicated process of steroid hormone formation

The whole body autoradiographic technique described by Ullberg (1954 1958) has been used to get the gross distribution of the C^{14} labelled precursors Detailed studies on the distribution in some selected organs have been made according to a modification of a method described earlier (Hammarstrom et al 1965) Quantitative determinations of C^{14} in some organs after injection of the labelled substances have been carried out as a complement to the auto radiographic investigation Some chromatographic studies have been made in order to elucidate what the majority of the C^{14} activities in the autoradiograms represents in the different cases The sites of steroid hormone formation were also studied by histochemical localization of hydroxysteroid dehydrogenase activity in whole body sections from mice A preliminary report of this work has been presented (Appelgren 19 6

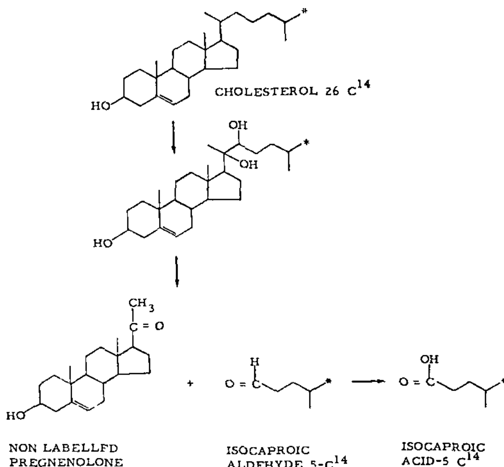


Fig › Postulated pathway for the side chain cleavage of C 26 cholesterol resulting in non labelled steroids and labelled isocaproic acid The asterisk indicates the site of labelling

MATERIALS AND METHODS

Whole body autoradiography

C^{14} labelled cholesterol was obtained from The Radiochemical Centre Amers ham England The C^{14}-4 cholesterol had a specific activity varying between 19 4 and 24 6 mci/mM and the C^{14} 26 cholesterol had a specific activity of 24 0 mci/mM

Radiochemical purity was tested with thin layer chromatography with cyclohexane and ethylacetate (50 50) as a solvent system The different spots which were located by autoradiography of the chromatogram were scraped off with a razor blade and transferred to counting vials containing 10 ml 0 5 % PPO (2 5 diphenyloxazole Packard) in toluene The radioactivity of

the spots with the same R_f values as the standard inactive cholesterol was calculated as per cent of the total counts of the scrapings from the origin to the front More than 97 % had an R_f value corresponding to that of inactive cholesterol

Tests were made to check if different amounts of silica gel caused quenching No differences between applied standard spots (about 5 mm diameter) could be seen in the counts when squares up to 2×2 cm were scraped off the plates and counted together with the spot Further different concentrations of C^{14}-cholesterol spotted on silica gel plates gave analogous results with the standard samples

C^{14}-4 cholesterol Thirty white mice (NMRI) 19 adult female 7 adult male and 4 pregnant female mice were used in the experiment with C^{14} 4 cholesterol The female and male mice weighed between 18 and 23 g and the pregnant ones 25 to 35 g The pregnant mice were injected 7 days (1 animal) 14 days (1 animal) and 18 days (2 animals) after mating as determined by observation of vaginal plugs Sixteen of the animals 10 female 3 male and 3 pregnant mice were injected intramuscularly or subcutaneously with the substance dissolved in arachidic oil Eleven animals 6 female 4 male mice and 1 pregnant mouse (late gestation state) were injected intravenously in a tail vein with the substance dissolved in ethanol Twenty five microliters of the ethanol solution were given to each of the animals Disposable 25 μl pipettes (Microcaps® Drummond Scientific Co Broomall Pa USA) were used and attached to a syringe with a polyethylene tubing system containing isotonic saline solution The ethanol solution was then slowly injected with the aid of the saline solution

Three female animals were given the radioactive substance perorally dissolved in arachidic oil or as an emulsion The emulsion was prepared with the aid of Tween 80®

The doses varied between 2 μci and 5 5 μci corresponding to 0 03 mg—0 1 mg cholesterol

The intravenously injected female mice were sacrificed 2 20 60 minutes and 4 24 hours and 4 days after the injection and the intravenously injected male mice 60 minutes and 4 20 and 60 days after the injection The intravenously injected pregnant mouse was killed 60 minutes after the injection The animals given the cholesterol orally were allowed to survive for 60 minutes

Among the subcutaneously or intramuscularly injected animals the survival times were for 2 series of female mice 60 minutes 4 24 hours 4 and 10 days One mouse sacrificed 24 hours after injection was also injected simultaneously with chorion gonadotropic hormone (12 IU Pregnyl® Pharmacia Sweden) The male mice were sacrificed after 4 24 hours and 10 days The pregnant mice in different gestation states were sacrificed [illegible] minutes after the subcutaneous injection

C^{14}-26-cholesterol Twenty-two white mice (NMRI) 11 adult female 7 adult male and 4 pregnant female mice were used The female and male mice weighed between 18 and 20 g and the pregnant ones 25 to 35 g The pregnant mice were injected 7 days (1 animal) 14 days (1 animal) and 18 days (2 animals) after mating

Eleven of the animals 5 female 3 male and 3 pregnant mice were injected intramuscularly or subcutaneously with the substance dissolved in arachidic oil

Eleven animals 6 female 4 male and 1 pregnant mouse (late gestation state) were injected intravenously as described earlier The doses varied between 2 μci and 5 5 μci corresponding to 0 03—0 09 mg cholesterol The different survival times were for the intravenously injected female mice 2 20 60 minutes and 4 24 hours and 4 days and for the male ones 60 minutes and 4 20 and 60 days and the pregnant mouse 60 minutes

The female mice subcutaneously or intramuscularly injected were sacrificed after 60 minutes 4 hours 4 and 10 days The male mice were sacrificed after 4 24 hours and 10 days The pregnant mice in different gestation states were sacrificed 60 minutes after the subcutaneous injection

The animals were anaesthetized with ether before being sacrificed by immersion in hexane cooled to — 78 C The mice were embedded in a mixture of carboxymethyl cellulose and water Sagittal sections (20 μ and 60—80 μ) of the whole body were cut and dried at — 10 C according to the Ullberg autoradiographic technique by which each section is attached onto tape (No 810 Minnesota Mining and Manufacturing Co) (Ullberg 1954 1958) The tape mounted sections were pressed against X ray films and stored in press at — 10° C For 20 μ thick sections Structurix (Gevaert) and for 60—80 μ thick sections Kodirex (Kodak) were used In order to avoid the artefacts sometimes seen in autoradiograms of fat soluble substances due to the melting of fat at room temperature all dark room work except developing was carried out at — 10 C Efforts have been made to avoid the unpleasant dark room work in a freeze room by treating the sections with osmic vapors The results were not satisfactory for cholesterol although this method has been shown to prevent blurring when working with some other fat soluble compounds (Appelgren unpublished observations) Fixation with Bouin s solution (Romeis 1948) gave somewhat better results but is was thought wiser to keep to the freeze room work rather than to rely on a fixation that might not be free from objections The exposure times varied from 15 days to 6 months Developing of the X ray films was done after the removal of the tape mounted sections in the freeze room Finally the sections were stained with hematoxylin and eosin and mounted on glass slides in Euparal® (Flatters & Garnett Ltd Manchester England)

Semiquantitative evaluation of the autoradiograms

To facilitate the quantitative evaluation within the autoradiograms and to reduce the magnitude of errors due to film quality and developing procedures isotope standards were prepared and apposed to the X-ray film together with each histological section during autoradiographic exposure.

Each standard consisted of 15 squares of different isotope concentration of C^{14} arranged in a series of decreasing activity such that adjacent members of the series were related in the ratio 1/2 forming a "staircase" of radioactivity. The staircases were made according to Berlin and Ullberg (1963). The evaluation of the autoradiograms was made by comparison with the naked eye between the different organs and the staircase. Prints were made simultaneously of both the mouse autoradiogram and the staircase. The staircase was cut off and placed on the autoradiogram and the step that coincided or was close to the blackening of the particular organ was notified. The 2 weakest steps were left without notice and the rest of the steps were called 1, 2, 2^2 (4), 2^3 (8) ... and 2^{12} (4096) for the strongest step.

Microautoradiography

Microautoradiography was performed according to Hammarström et al. (1965) with slight modification. From the intravenously injected female mice and from some of the male mice described above 5 to 10 micron thick sections were taken on tape (No. 688, Minnesota Mining and Manufacturing Co.). The sections were dried in the freeze room and then mounted onto G5 nuclear plates (10 μ thick, Ilford). This procedure was carried out in the freeze room to avoid the artefacts seen at room temperature as described earlier. The glycerin described in the original method was not used. The nuclear emulsion plates were left for exposure (20—60 days) under slight pressure. After exposure the plates were placed in xylene over night to dissolve the adhesive of the tape and were then passed through a series of absolute ethanol, 96 % ethanol and 70 % ethanol before photographic developing and fixing. After photographic processing and rinsing the sections were stained with hematoxylin and eosin, passed through a series of increasing concentrations of ethanol to xylene and finally mounted in Canada balsam. The autoradiograms and sections thus mounted together were then examined in the light microscope.

RESULTS

The distribution patterns from mice injected with C^{14}-26-cholesterol were very similar to those from animals given C^{14}-4-cholesterol.

In the following the distribution of C^{14}-4-cholesterol will be described and differences in the distribution patterns between the differently labelled substances will be pointed out.

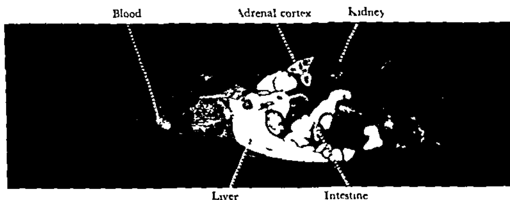


Fig. 3 Autoradiogram from a female mouse 4 hours after intramuscular injection of C^{14} 4 cholesterol Note the uptake (white areas) in the adrenal cortex that has not yet surpassed that of the liver

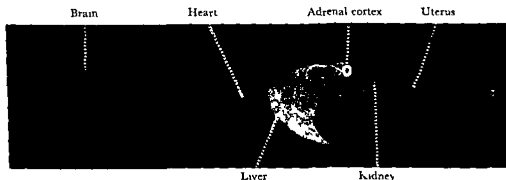


Fig. 4 Autoradiogram of a female mouse 24 hours after intramuscular injection of C 4 cholesterol Note the high uptake (light areas) in the adrenal cortex and the slight accumulation of C in the uterus

The route of administration of C^{14} cholesterol did not influence the distribution except in a few cases the digestive system showed of course higher concentration of cholesterol and/or its metabolites at the earlier times after oral administration than after parenteral injections The blood concentration was rather low and constant after subcutaneous and intramuscular injections and after oral administration After intravenous injection there was a rapid fall in the blood concentration during the first hours and then a small rise 4 hours after injection The blood concentration then slowly decreased but was still detectable 20 days after injection The differences seen in the lymphatic system will be described below

The most remarkable finding was the rather slow but intense accumulation of C^{14} cholesterol in the adrenal cortex The by far highest concentration in the body was seen there after 1 to 4 days after intravenous injection High uptake was also noticed in different parts of the ovaries and in the interstitial tissue of the testicles Sixty days after intravenous injection a high concentration of C^{14} was seen in the central nervous system and peripheral nerves

The semiquantitative evaluation of some organs in the autoradiograms with the aid of the isotope staircase is summarized in Tables 1 and 2

The distribution in the various organs will be described in detail below

The circulatory system

The *blood* concentration after intravenous injection of C^{14} cholesterol was lower than that of the liver and lung as early as 2 minutes after injection The myocardium showed less radioactivity than the blood at this time The concentration in the blood decreased rapidly after 20 and 60 minutes but a slight rise could be detected 4 hours after injection and then the blood concentration slowly decreased The *heart muscle* showed about the same level of C^{14} as the blood 24 hours after the injection Four days after intravenous injection of labelled cholesterol the concentration of the myocardium and the large vessel walls was higher than the blood and almost as high as in the liver After subcutaneous intramuscular and oral administration the blood concentration was rather low and constant during the experiment

The lymphatic organs

Four hours after subcutaneous injection of C^{14} cholesterol the red pulp of the spleen showed a concentration slightly higher than that of the blood The white pulp showed a slightly more intense uptake in the margin zone in most of the animals The lymph nodes and the thymus did not show any C^{14} at this time Twenty four hours after subcutaneous injection the red pulp of the spleen showed a concentration that was much higher than that of the blood The lymph nodes had some radioactive material located to their peripheral parts The thymus was still void of radioactivity at this time Four days after intramuscular injection the uptake in the spleen was the same as in the liver The lymph nodes appeared more homogenous at this time but the blackening did not exceed that of the blood very much

Two minutes after intravenous injection of labelled cholesterol the concentration was high in the spleen and the activity was confined to the red pulp with a distinct strong border line around the areas of white pulp The same distribution pattern in the spleen was observed 20 minutes 60 minutes and 4 hours after intravenous injections After 24 hours the cholesterol and/or its metabolites were evenly spread in the red pulp of the spleen and now some

Table 1 Semiquantitative evaluation of whole body autoradiograms after intravenous injection of C 4 cholesterol in mice The radioactivity in different organs has been compared with the autoradiogram of a simultaneously exposed C isotope staircase The staircase consisted of 15 steps the strongest represented by 2 (4096) and then forming a geometric series with the next step half the strongest one 2 (2048) and the next 1/4 2 (1024) and so on

	2 min	20 min	60 min	4 hours	24 hours	4 days	20 days	60 days
Blood	64	32	8	32	32	16	—	—
Myocardium	32	16	4	16	16	32	—	—
Spleen	256	512	256	128	128	32	—	—
Bone marrow	16	16	16	32	128	128	8	—
Skin	8	4	4	16	64	64	—	—
Intestinal walls	—	4	4	8	64	64	8	—
Liver	512	1 024	1 024	1 024	128	64	8	1
Kidney	16	4	4	16	64	32	16	—
Adrenal cortex	64	64	128	2 048	2 048	4 096	128	4
Brown fat	32	256	16	8	8	16	—	—
Testicle interst	—	—	—	—	256	512	32	—
Ovary corp lut	—	—	16	64	256	—	—	—
Ovary follicles	—	—	32	32	128	512	—	—
Ovary interst	—	—	16	16	16	256	—	—
Oviduct	—	—	8	16	—	—	—	—
Placenta	—	—	8	—	—	—	—	—
Brain white matter	—	—	—	—	—	—	8	16
Brain grey matter	—	—	—	—	—	—	—	2

Table 2 Semiquantitative evaluation of whole body autoradiograms after intravenous injection of C 26 cholesterol in mice The radioactivity in different organs has been compared with the autoradiogram of a simultaneously exposed C isotope staircase The staircase consisted of 15 steps the strongest represented by 2^{12} (4096) and then forming a geometric series with the next step half the strongest one 2^{11} (2048) and the next 1/4 2 (1024) and so on

	2 min	20 min	60 min	4 hours	24 hours	4 days	20 days	60 days
Blood	128	8	8	64	32	32	—	—
Myocardium	256	16	4	32	32	64	—	—
Spleen	64	64	128	256	256	64	—	—
Bone marrow	4	—	16	64	256	64	8	—
Skin	—	—	—	16	64	16	—	—
Intestinal walls	—	—	—	8	64	64	16	—
Liver	256	512	1 024	512	128	64	8	4
Kidney	8	—	—	64	—	128	16	—
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Oviduct	—	—	—	—	32	—	—	—
Placenta	—	—	16	—	—	—	—	—
Brain white matter	—	—	—	—	—	—	8	16
Brain grey matter	—	—	—	—	—	—	—	2

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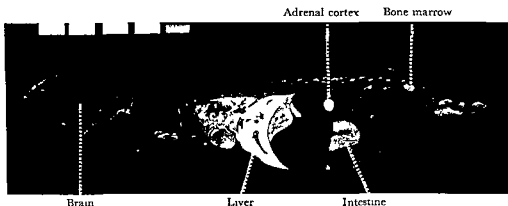


Fig 5 Autoradiogram of a female mouse 24 hours after an i v injection of C 4 chole terol Note the high uptake (light areas) in the adrenal cortex and the moderate uptake in the bone marrow The brain is void of radioactivity Note also the grey scale which is an auto radiogram of an isotope staircase consisting of small pieces of film that had been dipped in different concentrations of a C solution

pregnant animals variations in the uptake in the corpora lutea was apparent At early pregnancy (about 1 week) there were some very heavily labelled corpora lutea to be seen while animals in later pregnancy (> 2 weeks) showed about the same concentration in the corpora lutea as in the walls of the large follicles Four days after intravenous injection the interstitial tissue was heavily labelled and the difference between this tissue and the large follicles and corpora lutea was no longer so apparent (Fig 11) Some corpora lutea were sometimes weaker than other ones in non pregnant mice The corresponding sections showed that the weak corpora lutea were more eosinophilic than the highly active ones In most cases the follicle fluid and the eggs in large follicles showed some C^{14} The *oviduct* showed faint blackening mostly confined to the muscular layers 4 hours after administration of C^{14} cholesterol The muscular layers of the *uterus* showed the same concentration as the oviducts 24 hours after injection In the animal that received gonadotropic hormone the epithelial and glandular parts of the uterus showed about the same con centration as the interstitium in the ovary Four days after injection both the oviducts and the uteri showed rather low concentration of cholesterol and/or its metabolites

In a pregnant animal 60 minutes after injection of labelled cholesterol a rather weak blackening could be seen in the chorioallantoic *placenta* and in some animals the yolk sac epithelium was rather highly labelled The *fetuses* had very little radioacitvity during the experiment except in the liver where the concentration was about that of the placental blood 24 hours after injec tion

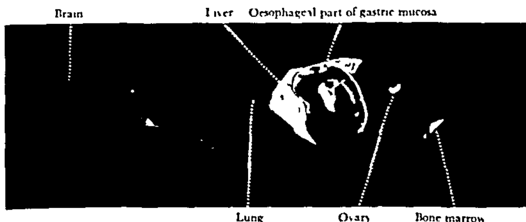
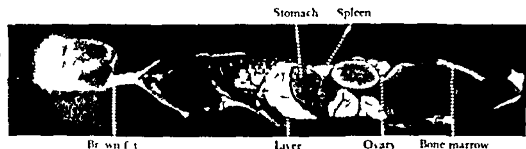
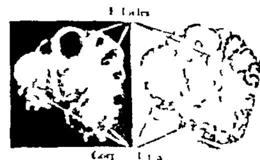


Fig. 6 Autoradiogram of a female mouse 24 hours after an i.m. injection of C[14]-4 cholesterol. Note the high uptake (white areas) in the ovary



Fi Autoradiogram from a female mouse 4 days after s.c. injection of C[14]-4 cholesterol. Note the high uptake (light areas) in the ovary



Fi 8 Detail of the ovary in Fig. and the corresponding section stained with hematoxylin eosin. Note the high uptake (light areas) in the walls of the large follicl

Male

Significant amounts of radioactivity could not be registered in the male sex organs until 24 hours after injection The *testes* showed a distribution pattern with the radioactivity localized in their interstitial parts Very weak radio activity was seen in the tubuli The *efferent ducts* and the *ductus epididymidis* were surrounded by rather strong radioactive streaks probably in the connective tissue and/or the muscle fibers Microautoradiograms showed that the epithelium had low concentration The accessory sex glands showed low amounts of C^{14} and the radioactivity was mainly surrounding the glands Four days after injection there was very high concentration in the interstitial cells of the testes and the tubular epithelium also showed some C^{14} (Fig 10)

The epididymis did not show higher amounts of cholesterol and/or its metabolites than the interstitia of the testis 4 days after injection as was the case 24 hours after the injection The epithelium of the accessory sex glands showed some radioactivity and about the same concentration could be seen in the penis surrounding the os penis and the cavernous spaces

As late as 20 days after injection the interstitial localization of C^{14} in testes was pronounced After 60 days rather weak concentration was noted A rather strong accumulation was noticed in the epithelium of the bulbo urethral glands

The nervous system

No significant amount of radioactivity could be detected in the *brain* until 20 days after injection when faint blackening could be noticed mainly localized to the white matter After 60 days there was a considerable accumulation in the white matter of the CNS compared with other organs in the body Also the grey matter showed some uptake of labelled cholesterol 60 days after injection (Fig 18 and 19)

Plexus choroideus had the same concentration as the blood in the beginning of the experiment but after 20 days a rather high concentration of C^{14} was observed there The cranial nerves showed a faint lining of radioactivity and so did the spinal ganglia and nerves leaving them After 20 days very strong uptake was noted in those places and still higher 60 days after injection After 4 days the *hypophysis* showed a marked uptake in its neural part while the glandular part showed a moderate concentration Pars intermedia of the pituitary wa void of radioactive material (Fig 17) This distribution pattern was also seen 20 days after injection

The *eye* showed very little C^{14} in the beginning of the experiment and the highest concentrations were localized to the cornea and the retina After 20 and 60 days the relative concentration in the eye increased in these parts









Fi ? U p p e r l e f t Micronautora liogram (section + autoradiogram) of an adrenal from a m use 4 day after an i v injection of C' 4 cholesterol Note the high and specific uptake of C (Black grains in zona fasciculata (A) The medulla (B) is void of radioactivity (5 nuclear emul i n Htx e n (x l)

F *10* U p p r r g h t Microautoradiogram (section + autoradiogram) of a testicle from a mou 4 day after i v injection of C' 4 cholesterol Note that the interstitial cells are stro gl labelle l black grain (nuclear emulsion Htx-eosin (x 3)

Fi *11* L w r l e f t Micr aut ra li gram (section + aut ra liogram) of an ovary from a mouse 4 f ft r nj ct n t C 4 holesterol The highest concentration of ((Black gra ns i see th nter tit um C r l i ul n Htx e in (x l()

Fig *12* L o w g h t Detail of F g w g a foll cle with high uptake of C' (Black grains) in bot t nul sa an l th a l

The digestive system

The *teeth* showed a low level of radioactivity in its pulpal and periodontal parts and this concentration was similar to that of the blood throughout the whole experiment The *salivary glands* showed very little radioactive material during all times studied but exceeded the blood concentration after 4 days and then remained fairly high until 60 days after injection The oesophageal parts of the *stomach* showed a very distinct localization of C^{14} 24 hours after injection but this disappeared after 4 days The highest concentration 4 hours after injection could be seen in the *liver* The bile showed very high amount of radioactivity and the secretion through the bile was apparently causing the rather high concentration seen in the lumina of the small intestines at this time

There was a fall of cholesterol and/or its metabolites in the liver after the peak at 4 hours and after 4 days the concentration did not surpass that of the blood very much The intestinal contents showed rather high to medium concentration through the whole experiment The intestinal walls showed more radioactive material than the blood 4 days after injection and this concentration was equal to that of skeletal muscle at that time The *pancreas* did not show any C^{14} during the first 10 days but after 20 days some radioactivity could be detected

C^{14} 26 cholesterol The excretion with the bile seemed to be less when C^{14} 26 cholesterol was given to the animals Thus 1 hour after intravenous injection of C^{14} 26 cholesterol the concentration in the bile was about the same as the blood concentration and after 24 hours to 60 days only weak blackening could be seen in the contents of the small intestines The walls of the intestine however showed a moderate accumulation There was a slightly higher concentration in the liver 20 days after injection of C^{14} 26 cholesterol than after injection of C^{14} 4 cholesterol and this difference was more pronounced 60 days after injection

The respiratory system

No radioactivity could be detected in the nasal mucosa or nasal secretion during the experiment The parenchyma of the *lung* showed a concentration corresponding to that of the blood 4 hours after injection Twenty four hours after injection there were more cholesterol and/or its metabolites in the lung tissue than in the blood Four days after injection the concentration in the lung was almost stronger than in the liver Neither the *trachea* nor the *bronchi* had any noticeable concentration throughout the experiment

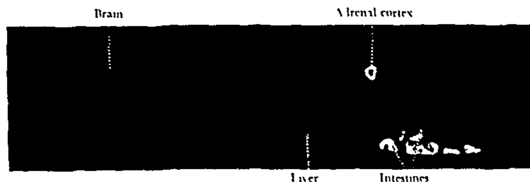
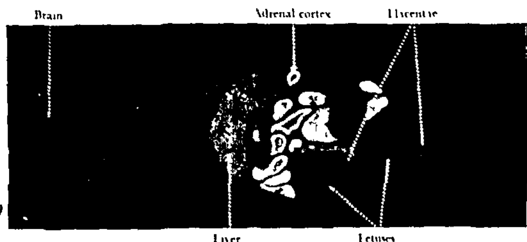


Fig 13 Autoradiogram of female mou e 4 days after an i m injection of C 4 cholesterol Note the high uptake (white areas) in the adrenal cortex



Fi 14 Autoradiogram from a mouse in late pregnancy 24 hours after i m injection of C 4 cholesterol Note the uptake (white areas) in the placenta The fetuses are void of radioactivity

The urinary organs

No C[14] could be detected in the *kidney* or *urinary bladder* 60 min subcutaneous or intramuscular injection Four hours after injectic radioactivity could be *seen in the cortex* of the kidney whereas th tubules and the hilus showed a little higher concentration bladder some radioactivity could be seen Twenty four h tion of labelled cholesterol there was a more even distrib of the kidney and the difference from the cortex was v In the cortex me spots blacke than the surround days after the njection the same pattern could be injection the above described p ppeared significant differences were observed

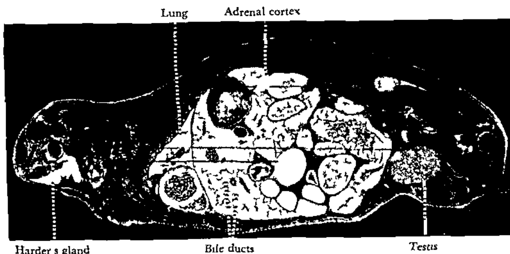


Fig 15 Autoradiogram from a male mouse 4 days after an i v injection of C 4 cholesterol Note the high uptake (white areas) in the interstitial parts of the testicle Note also that the adrenal is heavily overexposed

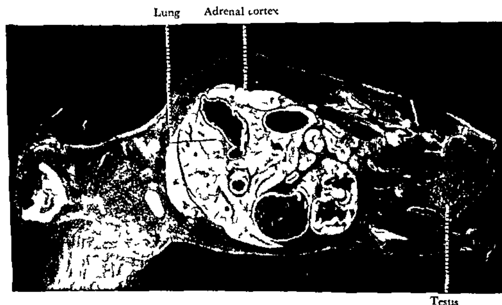


Fig 16 Autoradiogram from a male mouse 4 days after an i v injection of C 26 cholesterol Apparently the only difference in the distribution from Fig 15 is the higher concentration of C in the intestinal contents after injection of ring labelled cholesterol



Fig 13 Autoradiogram of female mouse 4 days after an i m injection of C 4 cholesterol Note the high uptake (white areas) in the adrenal cortex

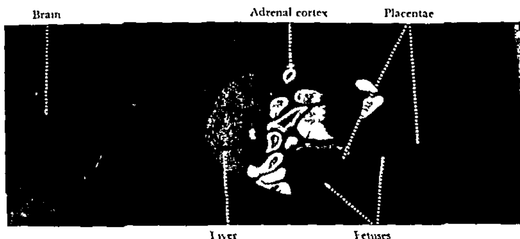


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The urinary organs

No C^{14} could be detected in the *kidney* or *urinary bladder* 60 minutes after subcutaneous or intramuscular injection Four hours after injection very little radioactivity could be seen in the cortex of the kidney whereas the collecting tubules and the hilus showed a little higher concentration In the urinary bladder some radioactivity could be seen Twenty four hours after the injection of labelled cholesterol there was a more even distribution in the medulla of the kidney and the difference from the cortex was very slight at this time In the cortex some spots blacker than the surrounding could be seen Four days after the injection the same pattern could be seen After intravenous injection the above described patterns appeared earlier but otherwise no significant differences were observed

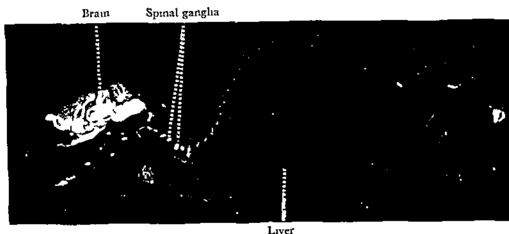


Fig 18 Autoradiogram of a male mouse 60 days after an i v injection of C 4 cholesterol Note the high uptake (light areas) in the brain and nerves The liver is almost void of C^{14}

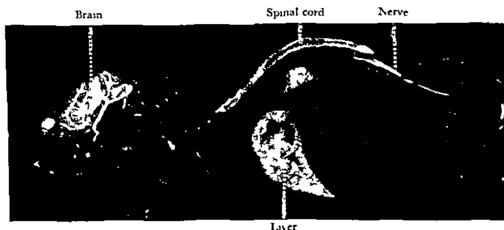


Fig 19 Autoradiogram of a male mouse 60 days after an i v injection of C 26 cholesterol Note the high uptake (light areas) in the brain and nerves The liver also shows some C

Muscles

The heart muscle showed weak C^{14} contents 4 hours after subcutaneous injection and the skeleton muscles were void of radioactivity at that time Twenty-four hours after injection the C^{14} level was still very low in the skeleton muscles but the heart had a concentration slightly less than that of the blood The heart muscle showed slightly more radioactivity 4 days after

Brown fat

Brown fat showed low concentration 2 minutes after intravenous injection of labelled cholesterol The concentration in brown fat was higher than in the blood 20 minutes after injection but after 4 hours these tissues showed the same concentration and this was the case even after 24 hours and 4 days After intramuscular injection the cholesterol and/or its metabolites in brown fat did not reach the blood level until after 24 hours

DISCUSSION

The uptake in endocrinologically active tissues after injection of C^{14} cholesterol will be discussed in General discussion in comparison with the distribution of the other steroid precursors studied but some remarks will be made here

Both the uptake and release of radioactivity after injection of C^{14} cholesterol in the adrenals ovaries and testes were slow in comparison with labelled pregnenolone and progesterone The distribution in these organs was the same for both ring labelled and side chain labelled cholesterol indicating that cholesterol and metabolites with the side chain intact were stored in these organs

Adrenals

The well known high concentration of cholesterol in the adrenal cortex in several mammals (Spector 1956 Kritchevsky 1958) was verified by the intense uptake of exogenous labelled cholesterol in the mouse adrenal cortex Four days after injection this accumulation was mainly confined to the zona fasciculata according to the microautoradiograms and most of the radioactivity then represented cholesterol in esterified form as shown by the chromatographic experiments Thus cholesterol esters localized in zona fasciculata may serve as a storage depot for the free cholesterol being the active substrate for steroidogenesis (cf Symington 1962 Goodman 1965 Davis and Garren 1966)

The small amount of radioactivity seen in the adrenal medulla 60 days after injection of C^{14} cholesterol may be correlated to the finding that chromaffin granules are rich in cholesterol (Blaschko et al 1966)

Ovaries

Earlier histochemical and polarization optical methods have shown heavy localization of cholesterol in the interstitial gland theca interna and corpora lutea in the ovaries in rabbits and rats (Claesson and Hillarp 1947a Claesson and Hillarp 1947b Claesson et al 1948) The present investigation with labelled cholesterol supports this finding but radioactivity was also found in

a low concentration in the granulosa cell layers and even in the ova The higher sensitivity of the present method may explain this difference

Testes

The specific uptake of labelled cholesterol in the interstitial cells of the testes agrees with earlier histochemical findings (cf Bourne 1958)

The nervous system

Chemical analyses of the nervous system show that there is a high amount of cholesterol (for review see Brante 1949) Next to the adrenal gland it is the tissue richest in sterol (Cook 1958) According to histochemical investigations with Schultz reaction the colour appears to be diffuse in the myelin The turnover of cholesterol in the brain is very slow C^{14} 4 cholesterol injected to newly hatched chickens (Davison et al 1958) and 17 days old rabbits (Davison et al 1959) has been shown to persist in measurable amounts for 150 days and one year respectively Furthermore the recovered cholesterol retained its radiocarbon in the original 4 C position indicating that myelin lipids may remain in the nerve sheaths where they were laid down originally during the development of the central nervous system

Chevallier and Petit (1966) fed grown up rats daily with C^{14} cholesterol in 8 or 60 days and sacrificed them after 60 days and autoradiographed the brains The white matter was heavily labelled in these autoradiograms

In the present investigation hardly any C^{14} was detected neither of C^{14} 4 cholesterol nor of C^{14} 26 cholesterol in the brain and spinal cord until 20 days after injection There was a considerable concentration in the white matter of the brain and in the nerves compared to the rest of the body 60 days after a single intravenous dose of labelled cholesterol No differences in the distribution were seen between the two differently labelled compounds indicating that unmetabolized cholesterol was taken up

The uptake of C^{14} around the nerves even at the early times studied indicated that the turnover of cholesterol there was more rapid than in the central nervous system

The pituitary

The pituitary showed an uptake of C^{14} earlier than in the brain after injection of labelled cholesterol The uptake of C^{14} was higher in pars nervosa than in pars distalis

The higher concentration of radioactivity in pars nervosa in the pituitary is in agreement with the finding that pars nervosa in ox pituitary had slightly more total steroids probably cholesterol than the rest of the pituitary (Mac Arthur 1919)

Brown fat

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Table 3 The table shows the variations in estrous cycles in six mice according to vaginal smears stained with acridine orange and studied in fluorescence microscope. These six animals were selected from a group of 14 because their cycles were most regular. The animals were all injected on the 30th of December and allowed to survive from 2 to 6 days. Some days vaginal smears were made both in the morning and in the afternoon.

Mouse No	Injected with	November 17	18	19	20	21	22	23	24	25		29	30 (Injection ↓)	December 1	2	3	4	5	6
9	C^{14}–26–chol	M	D	D	P E	M	M	M D	P E	M		M M	M	D	P –	–	– –	–	– –
13	»	P E	M	M	D P	E M	M	M	D	P		D M	M	P E	M	M –	– –	– –	–
12	»	M	M	M	D	P E	M	M	M	D		I E	M	M	M	D E	M	M	D
3	C^{14}–4–chol	M	M	D	D	P E	M	M D	M	I		M M	M	M	P –	– –	– –	–	– –
10	»	D	P E	M	M	D	P E	M	M	P		M M	D E	M	M	D	I I	M	M
5	»	M	D	P E	M	M	D	E M	M	D		D D	P E	M	D	D –	– –	– –	– –

M = metestrus D = diestrus P = proestrus E = estrus

DISTRIBUTION OF LABELLED CHOLESTEROL IN OVARIES OF MICE IN DIFFERENT PHASES IN THE ESTROUS CYCLE

The quantitative measurements after injection of labelled cholesterol showed that there was rather high and constant concentration of C^{14} in non selected ovaries from 24 hours to 10 days (p 33)

In order to study if variations in the estrous cycle had any effect on the distribution of labelled cholesterol within the mouse ovary the following investigation was made

MATERIALS AND METHODS

C^{14} 4 cholesterol and C^{14} 26 cholesterol were obtained from The Radio chemical Centre Amersham England with the specific activity of 24 6 mci/mM and 24 0 mci/mM respectively

Fourteen female NMRI mice were followed by vaginal smears during 14 days The vaginal smears were stained with acridine orange and the phase of the cycle was determined according to Vianney (1965) Six mice with regular sexual cycles were chosen for the experiment (Table 3) Three mice were injected intravenously with about 3 μci C^{14} 4 cholesterol and 3 mice with about 3 μci C^{14} 26 cholesterol corresponding to about 0 05 mg cholesterol The mice were followed with vaginal smears after the injection and sacrificed in different phases of the sexual cycle viz proestrus metestrus and diestrus The animals were allowed to survive for at least 2 days and not longer than 6 days

After the different survival times the animals were anaesthetized with ether and the right ovary from each animal excised through a small incision and immersed in fixing solution according to Bouin (Romeis 1948) This method was found to be the best according to preliminary experiments with whole body sections (p 9) Immediately after this procedure the whole animal was *frozen and subjected to whole body autoradiography as described earlier* The fixed ovaries were freeze sectioned and thawed onto slides and dried AR 10 stripping film (Kodak) was then applied on the sections (Doniach and Pelc 1950) and after drying the film bearing slides were stored in light tight boxes at + 4 C during the exposure After an exposure time of 20—50 days the autoradiograms were developed fixed and stained with hematoxylin and eosin Immediately before the sacrifice vaginal smears were taken from the animals and autoradiographed with AR10 stripping film as described above

The estrous variations in steroid contents of the interstitial gland in the rat ovary seen by Claesson and Hillarp (1947b) with histochemical methods were not observed with labelled cholesterol in mouse ovaries in this investigation

The distribution of labelled cholesterol in mouse ovaries in proestrus metestrus and diestrus showed very small variations and no definite differences could be registered

The same distribution pattern as described earlier for ovaries 4 days after an intravenous injection of labelled cholesterol was seen after 2 to 6 days Thus the interstitium was heavily labelled and the concentration in the walls of the large follicles was only slightly higher

The whole body autoradiograms were very similar when the two differently labelled substances were used which may indicate that the radioactivity represented cholesterol and/or metabolites with the side chain intact Chromatographic experiments indicated a slow esterification of the labelled cholesterol in the ovaries and not until 10 days after injection did most of the radioactivity represent cholesterol esters (p 38) This may explain the discrepancies between this investigation and that of Claesson and Hillarp (1947b) since Claesson et al (1948) observed that the concentration of esterified cholesterol varied much more than that of free cholesterol

The microautoradiographic examination gave identical results although the localization of isotope was not so distinct as could be expected from the whole body autoradiograms probably due to the melting of fat (p 9) The vaginal smears showed in some cases small differences The animals injected with C^{14} 4 cholesterol showed a somewhat higher number of labelled vaginal cells than the corresponding vaginal smears from animals injected with C^{14}-26 cholesterol which may indicate the formation of labelled estrogens However further experiments are required before any definite conclusions can be drawn

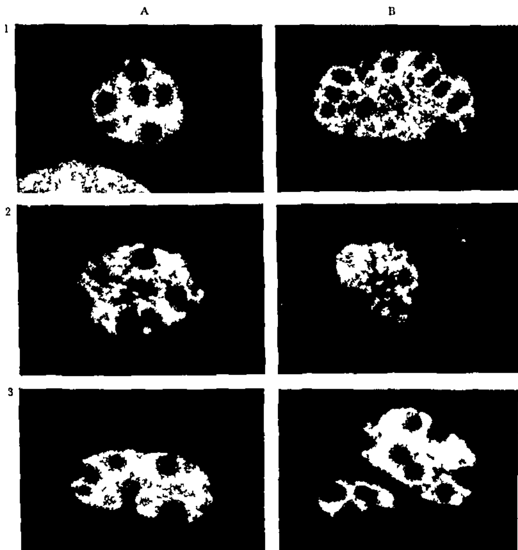


Fig 0 Details from whole body autoradiograms showing the distribution of C^{14} cholesterol (A) or C 26 cholesterol (B) in ovaries of mice in different phases in the estrous cycle

1 A Ovary from a mouse in proestrus 2 days after i v injection
2 A Ovary from a mouse in metestrus 6 days after i v injection
3 A Ovary from a mouse in diestrus 3 days after i v injection
1 B Ovary from a mouse in proestrus 2 days after i v injection
B Ovary from a mouse in metestrus 3 days after i v injection
3 B Ovary from a mouse in diestrus 6 days after i v injection

IMPULSE COUNTING OF C^{14}-CONTENTS IN VARIOUS TISSUES AFTER INJECTION OF C^{14}-4-CHOLESTEROL AND C^{14}-26-CHOLESTEROL

In order to get more quantitative results for some organs of C^{14} cholesterol injected mice the concentration of radioactivity in some organs was measured by impulse counting The comparison of the kinetics between cholesterol pregnenolone and progesterone was facilitated by this investigation since the autoradiographic studies mainly gave qualitative data

The distribution of C^{14} labelled cholesterol in the animal body as well as in humans has been studied earlier by impulse counting Gould (1952) gave blood containing labelled cholesterol to dogs and found that the specific activity of cholesterol in liver and spleen reached half that of plasma in a few hours and in kidney lung heart intestine and diaphragm in 1 to 2 days In the adrenal aorta and skin half the plasma value was reached more slowly Chevallier (1953) administered C^{14} labelled cholesterol orally to rats during several days After 9 days of feeding intestine liver and serum had high and essentially equal specific activity of cholesterol Spleen bone marrow lung depot fat heart and adrenal glands had between 60 and 80 % of the serum value and kidney skin and muscle about 30 % and the testicle somewhat less The brain had no detectable activity Borgstrom et al (1958) gave C^{14}-4 chol esterol orally to fasted rats and found that 50—70 % of the labelled cholesterol was rapidly absorbed in the intestinal mucosa and was delayed there before it was taken up in the chyle As a consequence of this delay of the absorbed cholesterol the blood activity curve reached its maximum about 16 hours after feed ing Avigan et al (1962) gave C^{14} 4 cholesterol rats and determined the ratios between specific radioactivities of cholesterol in various organs and that in serum as a function of time Values considerably above 1 were found in kidney and muscle after 20—49 days Values about 1 5 were found in liver lung heart spleen after 3 to 49 days In the brain values above 1 were reached after 49 days Chobanian et al (1962) made post mortem analyses of tissues for specific activity of C^{14} cholesterol in humans who died 1—226 days after receiving tracer doses of C^{14} labelled cholesterol Complete equilibration of serum and tissue cholesterol was seen within 1 month in liver spleen kidney lung adrenal intestine muscle abdominal aorta and fat In the brain the degree of equilibration was negligible Lossow et al (1962) injected C^{14} 27 cholesterol intravenously in rats After 24 hours they found the highest value in % of injected C^{14} per gram tissue in the adrenal and slightly less in spleen and liver Swell and Law (1966) studied the labelling of liver and serum cholesterol esters after injection of C^{14} 4 cholesterol and found a higher value in the liver than in serum after 24 hours

In the present study the adrenals ovaries uterus testes accessory sex glands blood and liver were selected for impulse counting The mice were injected intravenously with C^{14} 4 cholesterol or C^{14} 26 cholesterol and were killed at different intervals from 2 minutes to 20 days after injection The organs were then immediately removed and homogenized in saline solution and extracted in chloroform Aliquots of the extracts were counted in a Packard Tri Carb liquid scintillation spectrometer

MATERIALS AND METHODS

C^{14} 4 cholesterol and C^{14} 26 cholesterol were obtained from The Radiochemical Centre Amersham England with the specific activity of 24 6 mci/mM and 24 0 mci/mM respectively Radiochemical purity was tested as described earlier (p 7)

Two series of NMRI mice 16 male and 16 female mice were injected intravenously with either about 0 9 μci C^{14} 4 cholesterol or about 0 9 μci C^{14} 26 cholesterol The substances were dissolved in 25 microliters of ethanol and injected according to the method described earlier The animals weighed about 20 grams From each series 2 animals of each sex were allowed to survive for 2 20 and 60 minutes 4 and 24 hours 4 10 and 20 days The animals were sacrificed by stretching the spines From each animal samples of blood liver and adrenals were taken From the male mice testicles together with the epididymides and accessory sex glands (vesicular coagulating and ampullary glands and dorsal and ventral prostates) and from the female mice ovaries and uterus were taken The samples were pooled from the 2 animals with the same survival time and the wet weights were registered The tissue samples were homogenized in 1 ml 0 9 % saline solution in a Potter Elvehjem homogenizer and the lipids were then extracted with about 10 ml chloroform in 3 to 5 ml portions The chloroform phase was separated from the water phase by centrifugation and was sucked out with disposable pipettes and evaporated to dryness with a gentle stream of nitrogen The residue was dissolved in 1 ml chloroform and 0 1 ml samples were transferred to liquid scintillation vials containing a solution of 3 ml ethanol and 7 ml 0 5 % PPO in toluene and counted in a liquid scintillation spectrometer (Packard) From the supernatant in the water phase 0 1 ml was also counted in the same way The values were calculated as % gram dose per gram tissue The remaining tissue samples in the water phase were washed and dried and counted in a GM counter

RESULTS AND COMMENTS

The chromatographic control of the original substances showed that > 97 % had an R_f value corresponding to that of inactive cholesterol Hardly any radioactivity was found in the extracted tissue rests and will not be considered here The radioactivity of the water phases was low in blood liver and adrenals and varied between 0 and 3 % of the amounts found in the chloroform extracts In the sex organs the concentration in a few cases exceeded 3 % and values between 0 and 10 % were registered

The results of the quantitation of the chloroform phase are presented in diagrams in which the values in % gram dose/gram tissue are plotted against time after injection (Fig 21) The concentration of C^{14} between the two differently labelled compounds seemed to be almost identical after the different intervals after injection

The *blood* concentrations were high immediately after injection but decreased rapidly which might be due to the accumulation of injected C^{14} cholesterol in the liver After 20 minutes an increase of C^{14} in the blood was registered and this continued until a peak was reached between 4 and 24 hours Then a slow decrease in blood concentration was apparent The *liver* reached a peak between 1 hour and 4 hours and then slowly diminished and after 4 days the values slowly sank towards zero The decrease of labelled cholesterol in the liver coincided with the increase in blood concentration The differences in liver concentration between C^{14} 4 cholesterol and C^{14} 26 cholesterol seen in the autoradiograms from male mice 20 and 60 days after injection may be supported by the slight difference in the concentration observed in the male mice in this investigation 10 and 20 days after injection The most striking uptake of C^{14} was seen in the *adrenal* with a peak after 24 hours to 4 days and then decreasing but remaining high in comparison with other organs

The C^{14} concentration in the *ovaries* followed the blood concentration pattern and reached a maximum between 4 and 24 hours but in contrast to that of the blood then remained rather high The time sequence for the *testes* was very similar but somewhat lower concentrations were noted It must however be remembered that the quantitative data on the whole ovaries and testicles do not give justice to the high specific concentrations seen in various parts of these organs in the autoradiographic investigation Thus the differences in concentration between blood and follicles and corpora lutea in ovaries and between interstitial cells in the testicles were greater than could be judged from the diagrams However for comparison between the different precursors the diagrams seem to give adequate information

These quantitative data for the adrenals ovaries and testes confirm the findings from the autoradiograms that both the uptake and release of radio

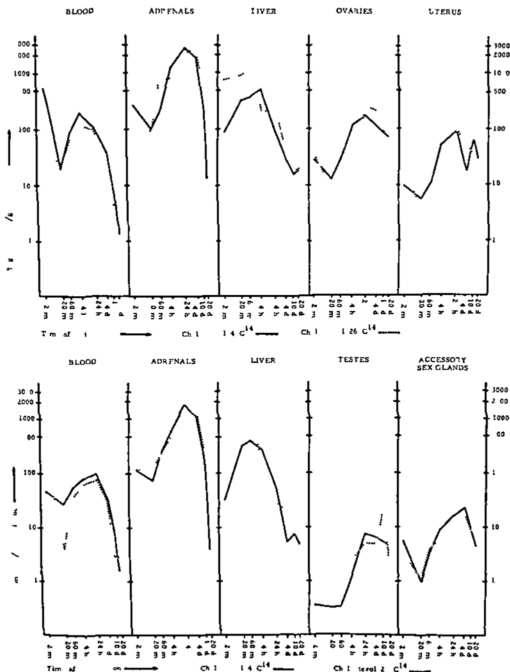


Fig 21 Radioa t ity in blood adrenals liver ovaries and uterus of female mice (upper) and blood adrenals liver testes and accessory sex glands of male mice (lower) at different intervals after i injection of C' 4 cholesterol or C' 26 cholesterol The ordinates give the contents pe gr m tissue as percentages of the dose given per gram animal Approximately the same doses of two d fferently labelled compounds were given

activity after injection of C^{14} cholesterol were slow in comparison with labelled pregnenolone and progesterone

In *uterus* and the *male accessory sex glands* also very good agreement between the two differently labelled compounds were seen This indicates that cholesterol as such was taken up there and may conceal a possible uptake of steroid hormones formed from ring labelled cholesterol

CHROMATOGRAPHIC STUDIES OF SELECTED TISSUES AFTER INJECTION OF C^{14}-4-CHOLESTEROL AND C^{14}-26-CHOLESTEROL

Cholesterol is metabolized in the body in different ways In the liver it has been extensively studied and known to be transformed to bile acids (for reviews see Bergstrom et al 1960 van Belle 1965) In steroid hormone producing glands it is transformed to various steroid hormones (cf Cook 1958 Kritchevsky 1958) Cholesterol is also esterified in the body to a great extent mainly with fatty acids (Cook 1958 Bowers and Schally 1965) Lately cholesterol sulphate has been demonstrated as an intermediate in metabolic pathways of steroid hormones (Roberts et al 1964 Lieberman 1966)

In order to find out what the majority of the radioactivity seen in the autoradiograms from animals injected with labelled cholesterol represented some chromatographic studies were made If possible the purpose was also to find out if the radioactivity in steroid producing organs represented intermediary metabolites such as pregnenolone and progesterone Of great interest was the question if labelled hormones were present in target organs e g estrogens in the uterus

In this study chromatographic investigations have been made on acetone ethanol extracts from various organs in animals injected with C^{14}-4 cholesterol or C^{14} 26 cholesterol and sacrificed after different survival times

MATERIALS AND METHODS

C^{14} 4 cholesterol and C^{14} 26 cholesterol were obtained from The Radiochemical Centre Amersham England with the specific activity of 24 6 mci/mM and 24 0 mci/mM respectively Radiochemical purity was tested as described before (cf p 7) and > 97 % of the radioactivity had the same R_f value as inactive cholesterol Two series of NMRI mice consisting of 3 female mice and 1 male mouse were used for the experiment The animals of one series were injected intravenously with C^{14} 4 cholesterol and the other series with C^{14} 26-cholesterol with the substance (about 1 μci) dissolved in 25 μl ethanol as described earlier (cf p 8) The mice weighed about 20 grams The survival times for the male mice were 4 hours and for the female mice 2 minutes 4 hours and 4 days The animals were sacrificed by stretching the spines and the following samples were taken blood liver adrenals ovaries or testicles and uterus or accessory sex glands (vesicular coagulating and ampullary glands and dorsal and ventral prostates) The tissues were immediately weighed and transferred to freeze box after adding 1 ml acetone ethanol (50 50) to

each sample The samples were then homogenized in a Potter Elvehjem homogenizer, filtered and extracted twice with the acetone ethanol mixture Preliminary experiments had shown that there was very little radioactivity left in the tissue rests when measured with GM counter The acetone ethanol extracts were evaporated to dryness with N_2 The residue was dissolved in 1 ml chloroform and 0 1 ml was counted in a liquid scintillation spectrometer (Packard) as described before (cf p 31) to determine if the samples were strong enough to give measurable chromatograms

Chromatography was made on thin layer silica gel G plates according to Stahl (1962) The silica gel layer was 250 μ and the plates were activated by heating at + 120 C for 30 minutes The front was allowed to run 15 cm After preliminary experiments the following solvent systems were used for developing the chromatograms (cf Stahl 1962)

I Cyclohexane diethylether acetic acid (30 20 2)
II Chloroform
III Carbon tetrachloride chloroform (96 4) Run 3 times
IV Methylene chloride acetone (80 20)

For obtaining R_f values of cholesterol and some possible metabolites the following reference substances were used C^{14} 4 cholesterol and C^{14} 26 cholesterol (The Radiochemical Centre Amersham England) cholesterol pregnenolone progesterone dehydroepiandrosterone deoxycorticosterone corticosterone cortisol cortisone cholesterol palmitate cholesterol oleate and cholesterol linoleate (Sigma Chemical Company Missouri USA) cholesterol acetate and cholesterol stearate (Preparations Laboratories Inc N Y USA) testosterone and estradiol (AB Leo Hälsingborg Sweden) The R_f values obtained for the reference substances in the systems used are shown in Table 4 Since variations of the R_f values were seen reference substances were run on the same plates as the radioactive tissue extracts in order to facilitate the comparison

The chromatograms of the non radioactive substances were made visible by spraying the plates with o phosphoric acid (1 70)-water (1 1) After heating the plates for 20 minutes at + 100° C they were studied in daylight and in ultra violet light

The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner (Packard) and/or by autoradiography The X ray film used for autoradiography was Kodirex (Kodak) The relative concentrations of the different radioactive spots were determined by scanning the chromatograms in a radiochromatogram scanner (Packard) When the radio activity was too low to be registered with the scanner the radioactive spots which were localized by autoradiography were scraped off and counted in a liquid scintillation counter (Packard) as described earlier (cf p 7) Characteri

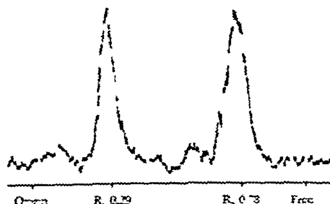


Fig. 2. Diagram of a radioactive thin layer chromatogram scanned in a radiochromatogram scanner (Packard). Tissue extract from adrenals from an animal 4 hours after injection of C^{14}-4-cholesterol was chromatographed in cyclohexane-diethylether acetic acid (90:20:2). Two peaks with R_f values corresponding to cholesterol (0.29) and cholesterol esters (0.78) were registered.

zation of some substances besides comparing them with known reference substances were made in a few cases. The radioactive spots with an R-value corresponding to that of cholesterol were scraped off and acetylated. The radioactivity was dissolved from the silica gel with chloroform, filtrated and evaporated to dryness with N_2. The residue was dissolved in pyridine and a few drops of acetic anhydride were added and the mixture was left overnight in room temperature. Excess acetic anhydride was decomposed with crushed ice and the precipitated acetate collected by centrifugation, washed with a few drops of ice cold ethanol and dried in vacuo. The acetate was chromatographed in systems I and II and the distribution of radioactivity determined by scanning or by autoradiographs. The radioactive spots with R_f-values corresponding to those of some cholesterol esters were scraped off and hydrolysed in 50% KOH. They were then chromatographed in system I and the distribution of radioactivity controlled with the scanner or with autoradiography.

RESULTS AND COMMENTS

The quantitative evaluation of the acetone-ethanol extracts showed good agreement with the chloroform extracts when compared as % gram dose/gram tissue.

Extracts from blood, livers and adrenals (except the short survival times) had sufficient amount of radioactivity to be chromatographed and scanned with the radiochromatogram scanner, while extracts from the sex organs were chromatographed and analysed by autoradiography.

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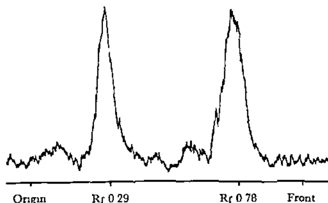
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Fig. 2° Diagram of a radio active thin layer chromatogram scanned in a radio hromatogram scanner (Packard) Tissue extract from adrenals from an animal 4 hours after injection of C^{14} cholesterol was chromatographed in cyclohexane diethylether acetic acid (30 70 2) Two peaks with R_f values corresponding to cholesterol (0 29) and cholesterol esters (0 78) were registered



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RESULTS AND COMMENTS

The quantitative evaluation of the acetone ethanol extracts showed good agreement with the chloroform extracts when compared as % gram dose/gram tissue

Extracts from blood livers and adrenals (except the short survival times) had sufficient amount of radioactivity to be chromatographed and scanned with the radiochromatogram scanner while extracts from the sex organs were chromatographed and analysed by autoradiography

Two main radioactive spots were seen in most of the chromatograms run in system I with R_f-values corresponding to cholesterol (0 27—0 30) and cholesterol esters (0 75—0 80) When the radioactive substance(s) with an R_f value of 0 27—0 30 was/were acetylated and chromatographed and auto radiographed only one radioactive spot with an R_f-value corresponding to that of standard cholesterol acetate appeared When the radioactive substances corresponding to cholesterol esters were hydrolysed the bulk of the radioactivity (> 90 %) appeared with an R_f value corresponding to that of cholesterol when chromatographed in solvent system I The rest of the radioactivity had the same R_f value as the original spot (0 80) which might be due to incomplete hydrolysis of the presumed cholesterol esters

The quantitative relation between the two main radioactive spots seen in system I varied with time and different tissues These variations for blood liver and adrenals are presented in Table 5 The fraction of presumed esterified cholesterol increased with time in all these organs

When some spots corresponding to cholesterol esters in system I were rechromatographed in system III and scanned and/or autoradiographed both quantitative and qualitative differences were noted between the different organs (Fig 23) Thus in the material from the blood 2 strong spots with R_f 0 33 and 0 42 and 3 weak spots with R_f 0 29 0 48 and 0 52 were seen

In the material from the adrenals one strong spot (R_f 0 39) and 3 weak spots (R_f 0 33 0 52 and 0 57) were seen

In the liver one strong (R_f 0 57) and 4 weak spots (R_f 0 37 0 42 0 52 and 0 62) were seen No definite identification of the presumed cholesterol esters could be done notwithstanding carrier substances were added in a few cases The carrier substances were cholesterol acetate linoleate palmitate oleate and stearate Cholesterol arachidonate known to occur in rats (Bowers and Schally 1965) has not been available However the present study showed that differences occurred between the blood and tissue cholesterol esters after injection of labelled cholesterol This is in agreement with other investigations which have shown that the relative distribution of different cholesterol esters in blood and tissue varies (Lossow et al 1962 Bowers and Schally 1965 Swell and Law 1966)

The extracts from the sex organs of the animals with a survival time of 4 hours were chromatographed in system I and autoradiographed More than 90 % of the radioactivity had an R_f 0 31—0 35 i e corresponding to standard cholesterol in testes/ovaries and male accessory sex glands/uterus from animals injected with C^{14}-4 cholesterol or C^{14} 26 cholesterol (Fig 24) Less than 1 % remained at the origin and the rest of the radioactivity had an R_f 0 80—0 83 probably cholesterol esters Further studies have shown that after 10 and 20 days about 90 % of the radioactivity in the ovaries had an R_f corresponding to cholesterol esters and the rest had an R_f corresponding to cholesterol

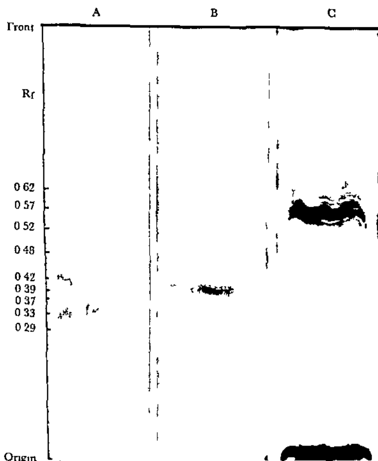


Fig 23 Radiochromatograms from tissue extracts from

A blood of an animal 4 hours after injection of C[1] [9]6 cholesterol The original tissue extract was first run in cyclohexane diethylether acetic acid (30 20 2) and the spots with R_f 0 75—0 80 were scraped off and chromatographed in carbon tetrachloride chloroform (96 4) 3 times Two strong spots (R_f 0 33 and 0 4?) and three weak spots (R_f 0 ?9 0 48 and 0 5?) are visible

B adrenals of an animal 4 days after injection of C[1] 4 cholesterol The original tissue extract was first run in cyclohexane diethylether acetic acid (30 20 ?) and then the spots with R_f 0 75—0 80 were chromatographed in carbon tetrachloride chloroform (96 4) 3 times One rather strong spot (R_f 0 39) and three weaker spots (R_f 0 33 0 52 and 0 57) are visible

C liver of an animal 4 hours after injection of C 4 cholesterol The original tissue extract was chromatographed in carbon tetrachloride chloroform (96 4) 3 times One strong spot (R_f 0 57) besides the origin and four weak spots (R_f 0 6? 0 52 0 4? and 0 37) are visible Exposure times for the radiochromatograms were about 3 months

Table 4 R_f values obtained for different reference substances in the following systems

I cyclohexane diethylether acetic acid (30 20 2)
II chloroform
III carbon tetrachloride chloroform (96 4) Run 3 times
IV methylene chloride acetone (80 20)

	I	II	III	IV
C^{14} 4 cholesterol	0 35	—	—	—
C^{14} 26 cholesterol	0 33	—	—	—
cholesterol	0 27—0 30	0 ?8	0 0?	0 57
pregnenolone	0 22	0 0 —0 14	0	0 47
progesterone	0 18	0 13—0 17	0	0 69
dehydroepiandrosterone	0 19	0 0 —0 12	—	0 46
corticosterone	0 1	0	—	0 10
cortisol	0	0	—	0 10
cortisone	0	0	—	0 13
cholesterol palmitate	0 80	0 81	0 52—0 61	—
cholesterol oleate	0 84	0 85	0 51	0 93
cholesterol linoleate	0 78	0 83	0 39	0 93
cholesterol acetate	0 66	0 73	0 16—0 23	—
cholesterol stearate	0 81	0 86	0 5?—0 70	—
testosterone	0 22	0 03—0 08	—	0 42
estradiol	0 10	0 06	—	0 45

Table 5 Quantitative relation of radioactivity between the 2 main spots seen after chromato graphy in system I (cyclohexane diethylether acetic acid 30 20 2) of extracts from blood liver and adrenals from mice injected with C^{14} 4 cholesterol or C 26 cholesterol The values are calculated as a percentage of total radioactivity spotted on the chromatographic plate The spots with R_f 0 27—0 30 and R_f 0 75—0 80 are supposed to represent cholesterol and cholesterol esters respectively

		C 4 cholesterol		C 26 cholesterol	
		R_f 0 27—0 30	R_f 0 75—0 80	R_f 0 27—0 30	R_f 0 75—0 80
Blood	2 min	95	5	95	5
	4 hours	70	30	71	29
	4 days	59	41	72	28
Liver	2 min	100	0	100	0
	4 hours	85	15	88	12
	4 days	45*	48	53**	24
Adrenals	2 min	—	—	—	—
	4 hours	50	50	—	—
	4 days	10 **	76	8	92

The following additional spots were also measurable

* Rf = 0 7
** Rf = 0 04 1 Rf = 0 56 7 Rf = 0 64 15
*** Rf = 0 05 9 Rf = 0 19 5

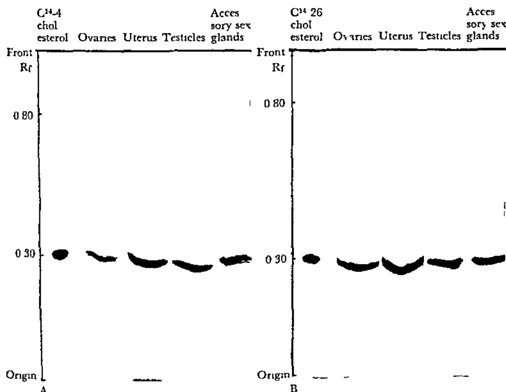


Fig 24 Radiochromatograms from tissue extracts from ovaries uterus testicles and accessory sex glands from animals 4 hours after injection of C^{14} 4 cholesterol (A) or C^{14} 26 cholesterol (B) According to quantitative measurements > 90 % of the radioactivity had the same R_f as the injected substance Very weak spots with $R_f \approx 0\ 80$ probably cholesterol esters are visible in some chromatograms Solvent system cyclohexane diethylether acetic acid (80 20 1) Exposure time 90 days

Conclusion

This investigation has indicated that practically all the radioactivity in the acetone ethanol extracts from some endocrinologically active tissues (adrenals ovaries testicles uterus and male accessory sex glands) from animals 4 hours after injection with C^{14} 4 cholesterol or C^{14} 26 cholesterol most likely represented unchanged cholesterol and cholesterol esters With time the radioactivity in the presumed cholesterol ester fraction in adrenals and ovaries increased in comparison with the free cholesterol

DISTRIBUTION OF C^{14}-4-PREGNENOLONE IN MICE

Pregnenolone has been used in the treatment of rheumatoid arthritis because of less side effects than with corticosteroid treatment but the therapeutic effect was dubious (cf Sollman 1957) Pregnenolone has also been reported to have some sex hormonal actions but only in exceedingly high dosage (for review see Henderson et al 1950) The actions may have been due to secondary formed steroid hormones since pregnenolone has been shown to be transformed *in vitro* to e g progesterone testosterone and corticosteroids in a number of investigations (Kahnt et al 1961 Acevedo et al 1963 Shikita et al 1964 Ellis and Berliner 1965 Vinson 1966 Ahmad and Gower 1966) As pregnenolone is regarded an important intermediate in the synthesis of steroid hormones from cholesterol it would be of great interest to study if also pregnenolone accumulates in the sites of steroid hormone synthesis An autoradiographic whole body study was therefore made with C^{14} 4 pregnenolone and in addition a microautoradiographic study was made on some selected organs

MATERIALS AND METHODS

C^{14} 4 pregnenolone was obtained from The Radiochemical Centre Amersham England with a specific activity of 24 0 mci/mM Radiochemical purity was tested on silica gel G using chloroform as a developing agent and > 98 % of the radioactivity had the same R_f value as inactive pregnenolone The substance was dissolved in ethanol and 25 microliters corresponding to 2 μci *were injected intravenously in 15 mice as previously described* Five female 5 male and 5 pregnant mice of the NMRI strain were used The animals in each group were allowed to survive for 5 20 and 60 minutes 4 and 24 hours The pregnant mice were in a late gestation state Whole body autoradiography was carried out according to Ullberg (1954 1958) as described earlier For microautoradiography 5 μ thick sections were taken from some of the above mentioned animals on tape 688 (Minnesota Mining and Manufacturing Co) and dry mounted onto G5 nuclear plates as described earlier (cf p 10) Such sections were taken from the female animals with survival times of 5 minutes and 60 minutes and from the 60 minutes pregnant mouse

RESULTS

There was a rapid excretion of C^{14} 4-pregnenolone after intravenous injection and very little radioactive substance was left in the body after 4 hours Twenty four hours after injection the remaining radioactivity was mainly localized to the intestines

There was a rapid accumulation in some organs e g brown fat adrenal cortex and brain The radioactivity disappeared very rapidly from these organs but a low concentration of C^{14} was detected in the adrenals 24 hours after injection Higher uptake of C^{14} was seen in the corpora lutea from pregnant animals than in the corpora lutea from non pregnant animal In the testes the radioactivity was confined to the interstitial cells In the placenta radioactivity was seen in the giant cell trophoblast layer as well as in the yolk sac placenta a short time after injection An interesting finding was the high concentration that was observed in the bronchi

The distribution in various tissues will be described in detail below

The circulatory system

The *blood* concentration was low as soon as 5 minutes after injection of labelled pregnenolone *The heart muscle* and the walls of the large *vessels* showed slightly higher concentration at this short time Twenty and 60 minutes after injection the heart muscle level was similar to the blood level In the *lymphatic organs* only the red pulp of the spleen showed slightly higher radioactivity than the blood 5 minutes after injection Twenty and 60 minutes 4 and 24 hours after injection the lymphatic organs showed no difference from the blood Ductus thoracicus showed some labelled material 20 minutes after injection

The adrenal glands

The *adrenal cortex* showed a fairly high accumulation of C^{14} 5 minutes after injection (Fig 25) The radioactivity decreased rather rapidly but a small amount comparable to that of the liver was observed 24 hours after injection The radioactivity was rather evenly distributed in the adrenal cortex 5 and 20 minutes after injection After 60 minutes and 4 hours most of the radioactivity was confined to the outer zones i e zona glomerulosa and zona fasciculata After 24 hours the faint radioactivity was evenly distributed in the cortex The *adrenal medulla* was void of radioactivity

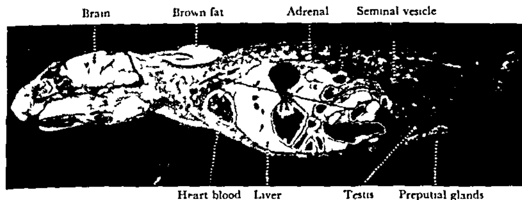


Fig 25 Autoradiogram of a male mouse 5 minutes after an i v injection of C[1] 4 pregnenolone Note the uptake (light areas) in the brain brown fat adrenal cortex and preputial glands

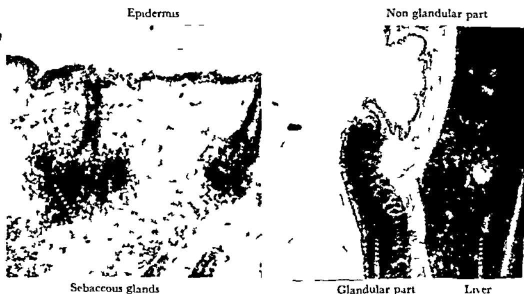


Fig 26 Left Microautoradiogram (section + autoradiogram) of the skin of a mouse 5 minutes after an i v injection of C[1] 4 pregnenolone Note the accumulation (black grains) in the sebaceous glands Htx eosin (x 75)

Fig 27 Right Microautoradiogram (section + autoradiogram) of the stomach of a mouse 5 minutes after an i v injection of C 4 pregnenolone Note the accumulation (black grains) in the glandular part of the stomach in comparison with the non glandular part Htx eosin (x 30)

The reproductive system

Female

Five minutes after the injection of C^{14}-4 pregnenolone the concentration in the *ovaries* from the non pregnant animal was higher than that of the blood The distribution of the radioactivity was even and leaving the contents of the large follicles empty In the ovaries of the pregnant animals (late gestation state) the corpora lutea showed somewhat higher accumulation than the rest of the ovary After 20 and 60 minutes there was a rather low concentration in the ovary of the non pregnant female However the follicle walls had a slightly higher concentration than the rest of the ovary One hour after injection the accumulation in the corpora lutea of the ovaries from the pregnant animal was almost as high as that of the liver (Fig 32) After 24 hours the corpora lutea also showed a concentration comparable to that of the liver The *oviduct* and *uterus* showed no marked uptake In the *placentae* an accumulation of C^{14} 4 pregnenolone and/or its metabolites could be seen 5 minutes after injection This concentration was observed around some blood lacunae probably in the giant cell trophoblast layer and to a smaller extent in the yolk sac epithelium The concentration in the labyrinth did not surpass that of the blood After 20 and 60 minutes rather high uptake was observed in the yolk sac epithelium while the rest of the placenta had the same concentration as the blood After 4 hours some C^{14} could be seen in only the yolk sac epithelium but after 24 hours also this part was void of radioactivity The *fetuses* showed very little evenly distributed C^{14} during the experiment The *mammary glands* showed a moderate concentration of C^{14} 4 pregnenolone and/or its metabolites throughout the experiment

Male

The interstitial parts of the *testes* showed strong concentration of C^{14} 4 pregnenolone and/or its metabolites 5 minutes after injection High amount of C^{14} was also seen in the epithelium of *ductus deferens* in the *preputial glands* and the epithelium of urethra possibly *urethral glands* (of Littré) Moderate activity was registered in the *epididymis* and the epithelium of the *vesicular glands*

Twenty minutes after injection there was high radioactive concentration in the interstitial parts of the testes The concentration in the epididymis was higher after 20 minutes than after 5 minutes The radioactivity in the epididymis was rather evenly distributed (Fig 29) The preputial glands were moderate in the accumulation of C^{14} 4 pregnenolone and/or its metabolite 20 minutes after injection Very little C^{14} was detected in the seminiferou tubules

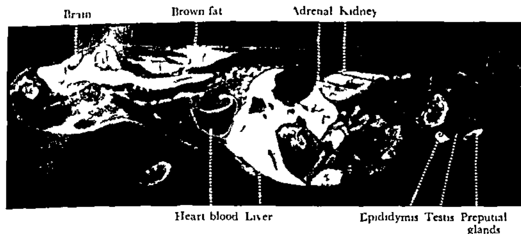


Fig 28 Autoradiogram of a male mouse 20 minutes after an i v injection of C^{14} 4 pregnenolone Note the uptake (light areas) in the brain adrenal cortex and testis

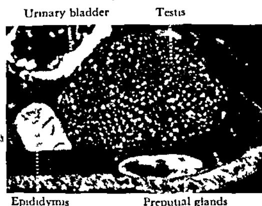


Fig 29 Detail of the testis in Fig 28 The uptake of C (light areas) is very pronounced in the interstitial cells of the testis In the seminiferous tubules very little radioactivity is visible Note the even distribution of C in the epididymis and the high uptake in the preputial glands

One hour after injection rather faint autoradiograms were seen of the male sex organs with the exception of the bulbo urethral glands which showed rather high concentration in the epithelium At later times no radioactivity could be registered

The nervous system

The *brain* and the *spinal cord* showed very high uptake of C^{14} 4 pregnenolone 5 minutes after injection (Fig 31) There was a slightly higher uptake in the grey matter than in the white matter Twenty minutes after the injection the concentration of C^{14} in the white matter exceeded that of the grey matter several times After 60 minutes no radioactivity could be detected in the grey matter while the white matter concentration was still rather high Four hours after the injection there was very low concentration in the white matter of the nervous system and after 24 hours no significant amounts of C^{14} were seen The peripheral nerves showed the same accumulation of C^{14} 4 pregnenolone as the white matter in the brain The ganglia showed very weak blackening in

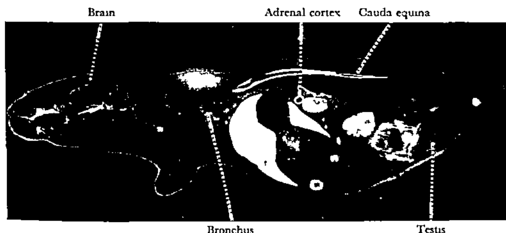


Fig 30 Autoradiogram of a male mouse 60 minutes after an i v injection of C 4 pregnenolone Note the uptake (light areas) in the brain bronchi peripheral nerves adrenal cortex and testis

Corpus callosum Cerebellum Corpus callosum Cerebellum

A B

C D

Corpus callosum Cerebellum Cerebellum

Fig 31 Details of brains from whole body autoradiograms (A B and C) of mice 5 (A) 20 (B) and 60 minutes (C) after v njection of C' 4 pregnenolone Note the uptake (light areas) in the grey matter 5 minutes after injection and the uptake in white matter 20 and 60 minutes after injection

D Detail of the bra n rom a whole body section of a mouse showing the histochemical localization of △ 3β I 1 oxysteroid dehydrogenase activity (dark areas) with pregnenolone as a substrate This enz activity is confined to the white matter (cf p 79)

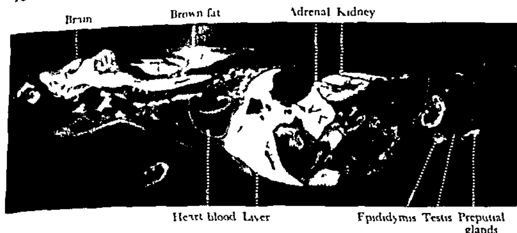


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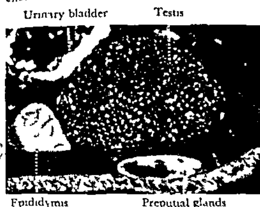


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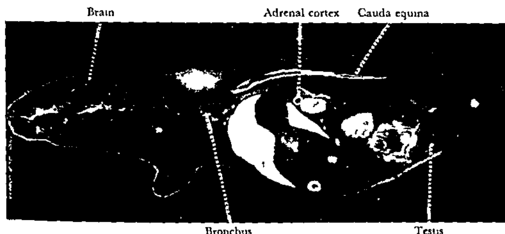


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the autoradiograms throughout the experiment Most of the radioactivity seen in the *eye* shortly after injection was localized to the retina

The digestive system

The *teeth* showed slightly higher radioactivity than the blood in their pulpal and periodontal parts 5 minutes after injection After 20 minutes the radioactivity in the above mentioned parts of the teeth was as low as the blood and 60 minutes after injection hardly any radioactivity could be detected

The *salivary glands* showed slightly higher concentration than the blood 5 minutes after injection but after 20 minutes there was no difference between the blood and the salivary glands In the *stomach* rather high radioactivity could be detected in the mucosa in fundus and moderate blackening was seen in the superficial layers of the oesophageal parts 5 minutes after injection (Fig 27) Later this accumulation was not pronounced and 20 minutes after injection some C^{14} could be seen in the gastric contents and this was true also after 60 minutes and 4 hours Twenty four hours after injection there seemed to be very high accumulation in the contents of the stomach The *small intestines* showed high concentration of C^{14} in their contents 5 minutes after injection The intestinal walls showed slightly higher blackening than the blood After 20 minutes there was very high concentration in the intestines The *liver* showed higher concentration of pregnenolone and/or its metabolites than the intestines 5 minutes after injection and this was the case even after 60 minutes The liver concentration was still high after 4 hours but then the intestinal contents showed the highest concentration in the body Twenty four hours after the injection little C^{14} could be detected in the liver The C^{14} concentration in the *bile* was very high all times after injection The *pancreas* showed about the same concentration as the heart muscle 5 minutes after the injection After 20 minutes there was no difference seen between the blood and the pancreas and later on no radioactivity could be registered

The respiratory system

The parenchyma of the *lung* showed the same C^{14} concentration as the heart muscle 5 minutes after intravenous injection of C^{14} 4 pregnenolone The large *bronchi* possibly the epithelium showed the same high concentration as the liver One hour after injection the bronchi and the liver showed the highest concentration in the body The lung parenchyma was void of radioactivity at that time After 4 hours there was still high concentration in the bronchi but after 24 hours very little could be detected

The urinary organs

The parenchyma of the *kidney* showed about the same concentration as in the liver 5 minutes after injection of labelled pregnenolone while the pelvis was

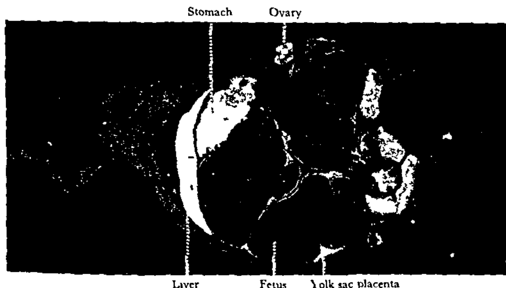


Fig 39 Autoradiogram of a pregnant mouse 60 minutes after an i v injection of C 4 pregnenolone Note the uptake (light areas) in the corpora lutea in the ovary and in the yolk sac placenta

void of radioactivity Twenty minutes after injection there was an increase in radioactivity in the medullary parts and the hilus of the kidney The concen tration in the cortex was rather low except in some streaks probably represent ing the collecting tubules At this time the *urinary bladder* showed a marked concentration The same distribution could be seen 60 minutes and 4 hours after the injection but after 24 hours hardly any radioactivity could be detected neither in the kidney nor in the urinary bladder

Hard tissues

In the *hard tissues* no C^{14} could be observed during the whole experiment In *bone marrow* the concentration was slightly higher than that of blood 5 minutes after injection but later on very little radioactivity was seen *Cartilage* showed no C^{14} In some cases a weak accumulation could be detected around the bones and joints probably in *connective tissue*

The muscles and skin

In the *skeletal muscles* the radioactivity was not evenly distributed In some muscle groups streaks could be seen 5 minutes after injection which had more C^{14} than the blood while most of the muscles had very little Twenty minutes after injection the concentration in the skeletal muscles was very low Hardly any radioactivity could be detected in the muscles 4 hours after injection

Five minutes after injection a strong spotty accumulation of C^{14} could be seen in the *skin* most likely localized to the *sebaceous glands* (Fig 26) In addition there was a non constant moderate uptake of C^{14} throughout the whole skin The appearance in the skin was the same after 20 and 60 minutes but after 4 hours the whole skin was very weak

Brown fat

There was an intense accumulation in the *brown fat* in the male mouse 5 minutes after injection of C^{14} 4 pregnenolone The female mouse showed less radioactivity and the pregnant female still less Twenty minutes after the injection the same concentration as in the brain was seen in all the animals and later on rather small amounts of radioactivity were detected

DISCUSSION

The uptake in endocrinologically active tissues after injection of C^{14} 4 pregnenolone will be discussed in more detail in the general discussion together with the distribution of the other steroid hormone precursors studied However some remarks will be made here

Adrenals testes and ovaries

In the adrenal cortex testes and ovaries the highest concentrations were seen 5 minutes after injection of C^{14} 4 pregnenolone and the concentrations were then decreasing This differs from labelled cholesterol which did not reach its highest concentration until 24 hours to 4 days after injection The difference may indicate a more rapid utilization of pregnenolone than cholesterol as a hormone precursor The chromatographic investigations support this theory since they have shown that most of the radioactivity in the testes and adrenal cortex 5 minutes after injection of labelled pregnenolone did not correspond to unchanged pregnenolone Some of the C^{14} metabolites seen may possibly be steroid hormones

The nervous system

The uptake of C^{14} 4 pregnenolone in the brain 5 minutes after injection is interesting since cholesterol did not reach a registerable concentration in the brain until 20 to 60 days after injection Short time after injection the C^{14} concentration was slightly higher in the grey matter than in the white matter a distribution pattern that was also seen for C^{14}-4 progesterone in the present investigation Progesterone is known to have an anaesthetic action while pregnenolone has been reported to have very slight effect (Selye 1941 Gyermek et al 1967) The distribution of labelled pregnenolone in areas rich in myelin

fibers after 20 and 60 minutes coincides with that of labelled progesterone after 10 minutes A similar localization in the white matter of the brain was observed after 24 hours in an autoradiographic study on C^{14} benzenehexa chloride an anticonvulsive agent by Koransky and Ullberg (1964) An anti convulsive action of pregnenolone has been reported by Spiegel (1946 1950) in cats In mice however this effect was negligible (Spiegel and Wycis 1945 Craig 1966)

The respiratory system

The high uptake of C^{14} 4-pregnenolone in the bronchi is difficult to explain Labelled progesterone was also shown to localize in the same place in the present investigation Vitamin C known to play some role in steroidogenesis (cf Degkwitz et al 1965) also localizes in the bronchi (Hammarström 1966) It is interesting to compare the localization of steroid hormone precursors as well as vitamin C in intact bronchial epithelium in mice with the suggestions of Goldman (1961) that bronchial carcinomata may be sites of estrogen synthesis in humans

Labelled cortisone and cortisol have also been demonstrated in the bronchi of mice by Hanngren et al (1964) and later Koerner (1966) found 11β hydroxysteroid dehydrogenase with cortisol as a substrate in the mouse lung

Skin

Estrogens and androgens are known to alter the size of sebaceous glands and to modify their secretory activity (Ebling 1948 Haskin et al 1953)

Baillie et al (1965) have shown that sebaceous gland hydroxysteroid dehydrogenases probably play a role in acne vulgaris in humans Against this background it is interesting to note the Δ^5 3β hydroxysteroid dehydrogenase activity in the sebaceous glands in mouse and the rather strong specific uptake of labelled pregnenolone in these glands observed in the present investigation

Brown fat

Brown fat showed high accumulation of labelled pregnenolone 5 minutes after injection in this investigation In a review on brown fat Johansson (1959) has pointed out that pregnenolone produces a marked increase in the specific gravity and disappearance of the lipid droplets in the brown fat The fact that there was a higher C^{14} concentration in the brown fat in the male than in the female mice in the present study may be put in relation to the findings of large amounts of androgens in brown fat (Sweet and Hoskins 1940) and the statements that brown fat continues to function longer in boys than in girls and that it probably secretes androgens (Pawlikowski 1955) Thus it may be possible that pregnenolone accumulates in brown fat as a precursor for androgens

CHROMATOGRAPHIC STUDIES AND QUANTITATIVE MEASUREMENTS OF SELECTED TISSUES AFTER INJECTION OF C^{14}-4-PREGNENOLONE

Pregnenolone is known to be transformed to steroid hormones in the body Regarding the formation of testosterone *in vitro* studies of the metabolism of pregnenolone have led to the suggestion that two pathways exist one involving progesterone as an intermediate and the other involving dehydroepiandrosterone (DHA) Kahnt et al (1961) found both 17α hydroxypregnenolone and DHA as metabolites of pregnenolone in bovine testicular tissue but Acevedo et al (1963) found only DHA in human fetal testis Shikita et al (1964) found that pregnenolone was transformed into testosterone in rat testicular microsomes Since labelled progesterone was found but only negligible quantities of 17α hydroxypregnenolone and DHA they concluded that testosterone is preferentially formed via progesterone in the rat However Ellis and Berliner (1965) showed an alternative pathway via 17α hydroxypregnenolone in mouse testes besides the formation of testosterone via progesterone

In rat adrenal glands Vinson (1966) has shown that the transformation of pregnenolone to corticosteroids has progesterone as an intermediate Δ^{16} steroids have been found in small quantities after incubation with C^{14} 4 pregnenolone and rat testes (Ahmad and Gower 1966) and boar testis and adrenal (Gower and Ahmad 1966)

In order to find out to what extent the radioactivity seen in the autoradiograms from animals injected with labelled pregnenolone represented unchanged C^{14} 4 pregnenolone some chromatographic studies were made Some of the metabolites mentioned above were used as reference substances

To be able to make a more quantitative evaluation of the autoradiographic pictures and to facilitate the comparison of the kinetics of cholesterol and pregnenolone measurements of the radioactivity in chloroform extracts from some organs of mice were made by impulse counting after injection of C^{14} 4 pregnenolone

MATERIALS AND METHODS

C^{14} 4 pregnenolone was obtained from The Radiochemical Centre Amersham England with a specific activity of 24 0 mci/mM Radiochemical purity was tested on silica gel G using chloroform as a developing agent and > 98 % of the radioactivity had the same R_f value as inactive pregnenolone Two series of NMRI mice consisting of 4 male and 4 female mice were used for the experiment Every one of the animals was injected intravenously with 2 μci

C^{14} 4 pregnenolone dissolved in 25 μl ethanol The mice weighed about 20 grams The survival times for the two series of mice were 5 20 60 minutes and 4 hours The animals were sacrificed by stretching the spines and the following samples were taken blood liver adrenals testes/ovaries male accessory sex glands/uterus brain and brown fat The epididymides were not separated from the testes The accessory sex glands were vesicular coagulating and ampullary glands and dorsal and ventral prostates The tissues were weighed and transferred to a freeze box after adding 1 ml of physiological saline solution to each sample The samples were then homogenized in a Potter Elvehjem homogenizer and extracted twice in 3 ml chloroform The chloroform phase was sucked out with disposable pipettes after centrifugation and evaporated to dryness with N The residue was dissolved in 1 ml chloroform

Impulse counting

0 1 ml of the chloroform extract and 0 1 ml of the water phase were counted in a liquid scintillation spectrometer (Packard) as described earlier (cf p 31) The tissue rests were washed twice with saline solution and the radioactivity in each sample measured with a GM counter (Tracerlab) The % gram dose per gram tissue of the chloroform extracts was calculated

Chromatography

Chromatography of the chloroform extracts and of a few samples of the water phases was made on thin layer silica gel G plates according to Stahl (1962) and as described previously (p 36) After preliminary experiments the following solvent systems were used

I Chloroform
II Chloroform acetic acid (90 10)

For obtaining R_f values of pregnenolone and some possible metabolites the following substances were used C^{14} 4 pregnenolone C^{14} 4 progesterone (The Radiochemical Centre Amersham England) pregnenolone progesterone 17α OH pregnenolone 17α OH progesterone dehydroepiandrosterone cortisone cortisol corticosterone deoxycorticosterone d aldosterone (Sigma Chemical Company Missouri USA) testosterone and estradiol (AB Leo Halsingborg Sweden) pregnenolone acetate and dehydroepiandrosterone acetate (synthetized according to the method described earlier p 37)

The R_f values obtained for the reference substances in the 2 systems used are shown in Table 6

Since variation of the R_f values were seen reference substances were run on the same plate a the radioactive tissue extracts in order to facilitate the comparison of R_f values

The chromatograms of the non radioactive substances were made visible by spraying the plates with o phosphoric acid (1 70) water (1 1) and heating them for 20 minutes at + 100° C and studied in ultra violet light

The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner and/or autoradiography as described before The relative concentration of each spot was determined by impulse counting in a liquid scintillation spectrometer (Packard) as described earlier

The chloroform extracts were chromatographed in both the systems when there was sufficient radioactivity In some cases the whole extract was used in one single spot to get enough radioactivity for detection The water extracts from the blood and liver were chromatographed in both the systems

Attempts to characterize the presumed pregnenolone and pregnenolone esters besides comparing them with reference substances were made in a few cases by acetylation or hydrolysis respectively (cf p 37)

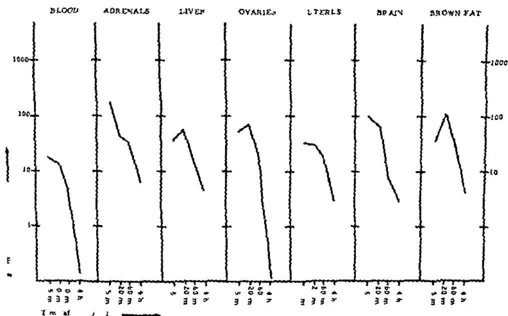
RESULTS

Impulse counting

The radioassays on the washed tissue rests showed that practically all of the radioactivity was extracted except in the liver samples which contained about 1 % of the total radioactivity in the organ The radioactivity in the chloroform extracts from the different organs was calculated as % gram dose per gram tissue and presented in diagrams (Fig 33) As can be seen from these diagrams the radioactivity in all the tissues studied decreased very rapidly which is in agreement with the autoradiographic findings However the total contents of C^{14} in most tissues are not shown by these diagrams since there were rather high amounts of C^{14} left in the water phases There was an increase of C^{14} with time in the water phases compared with the radioactivity in the chloroform extracts in all tissues studied In the water phase of the blood there was about 10 % 5 minutes after injection and about 40 % after 4 hours Of the C^{14} in the liver 40—60 % was left in the water phase and in the adrenals testes and ovaries there was about 10—30 % left In the brain and brown fat the radioactivity in the water phase was below 1 % 5 minutes after injection and then the value increased to about 8 % 4 hours after injection In uterus and accessory sex glands the corresponding values were 3—20 %

Chromatography

The radioactive metabolites in the water phases from the liver and blood remained at the start in both the systems used and no radioactive substances with an R_f corresponding to unchanged pregnenolone was observed Several radioactive metabolites were detected when the chloroform extracts from the



BLOOD ADRENALS LIVER TESTES ACCESSORY SEX GLANDS BRAIN BROWN FAT

1000
100
10
1

Time af inje on

Fig 33 Radioactivity in chloroform extracts from blood adrenals liver ovaries uterus brain and brown fat in female mice (upper) and blood adrenals liver testes accessory sex glands brain and brownt fat in male mice (lower) at different intervals after i.v. injection of C^{14} 4 pregnenolone The ordi es give the contents per gram tissue as percentages of the dose given per gram animal

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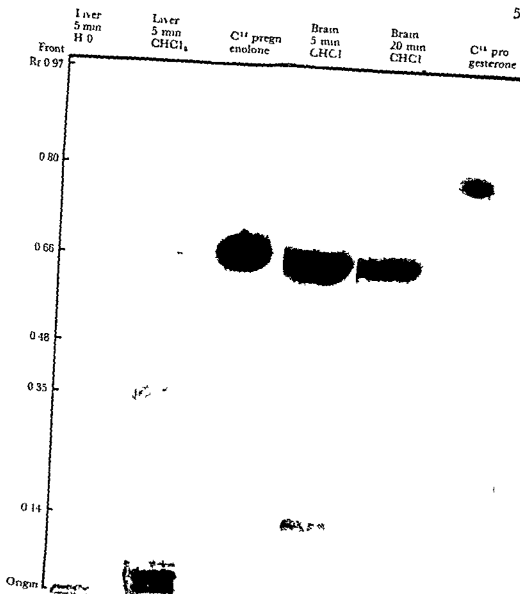


Fig 34 Radiochr m t g am of ti ue extracts of m ce and 0 minutes after an i v injec tion of C 4 p eg 1 lon The chlo ofo m pha e as w ll a the water phase from the live were chromatog ph e l No labelled metabol tes cor e p nding to pregnenolone were registered in the water ph (mp e Table fo quantitat ve comparison of the different metabolites System chlor f) etic acid (10 10)

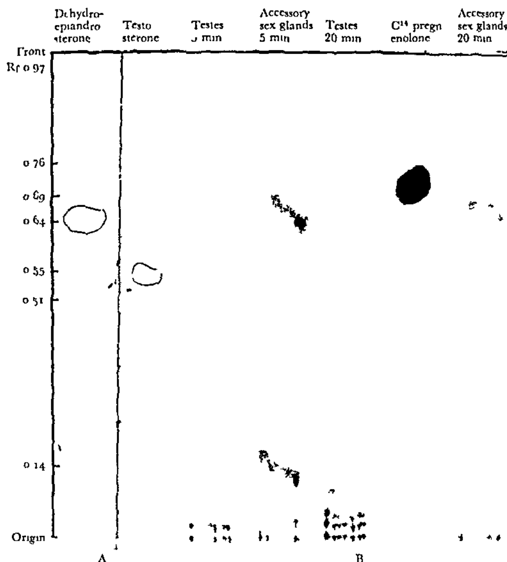


Fig 35 Radiochromatogram (B) of tissue extracts of mice 5 and 20 minutes after an i v injection of C^{14} pregnenolone Photograph (A) of non radioactive reference substances from the same plate Compare Table 7 for quantitative evaluation of the different metabolites Solvent system chloroform acetic acid (90 10)

Conclusion

A very rapid metabolism of pregnenolone was found As early as 5 minutes after injection less than 30 % of the C^{14} in the chloroform phase from blood testes and liver had R_f values corresponding to unchanged pregnenolone In brain brown fat and male accessory sex glands the corresponding values were 80 60 and 50 % respectively In the blood testes adrenals and brown fat labelled compounds which may represent steroid hormones were found

Table 6 R_f values obtained for different reference substances in the following systems

I Chloroform
II Chloroform — acetic acid (90 10)

	I	II
C 4 pregnenolone	0.04—0.08	0.65—0.74
C 4 progesterone	0.11—0.13	0.83
pregnenolone	0.07—0.14	0.64—0.70
progesterone	0.13—0.17	0.80
17α OH pregnenolone	0.03	0.45
17α OH progesterone	0.06—0.12	0.55
dehydroepiandrosterone	0.07—0.12	0.64—0.71
testosterone	0.03—0.08	0.56
estradiol	0.06	0.45—0.50
cortisone	0	0.06
cortisol	0	0.04
corticosterone	0	0.14
deoxycorticosterone	0.09	0.59
d aldosterone	0	0.14
pregnenolone acetate	0.52	0.89—0.96
dehydroepiandrosterone acetate	0.46	—

Table 7 Percentage of total radioactivity in different spots after chromatography of chloroform extracts from liver brain testes and accessory sex glands from animals 5 and 20 minutes after an i v injection of C 4 pregnenolone System chloroform acetic acid (90 10) The R value for C 4 pregnenolone was 0.65—0.74 and for testosterone 0.56 Compare Table 6 for R_f values of other reference substances used

	Liver		Brain				Testes		Accessory sex glands	
	5	20	5		20		5	20	5	20
	♂	♂	♂	♀	♂	♀	♂	♂	♂	♂
Rf 0.97	—	—	3	2	2	4	—	2	7	—
0.76	—	—	—	—	—	—	6	5	—	—
0.64—0.69	13	8	80	80	69	80	16	16	47	62
0.55	—	—	—	—	—	—	21	13	—	—
0.45—0.51	17	10	3	3	3	3	13	19	—	—
0.33—0.38	15	4	2	2	1	2	—	—	—	—
0.14	—	—	8	10	11	6			30	11
0.09—0.14	19	14	—	—	—	—	} 44	} 45	} 16	} 27
0 —0.05	36	64	4	3	14	5				

Table 8 Percentage of total radioactivity in different spots after chromatography of chloro form extracts from blood and brown fat from animals 5 and 20 minutes after an i v injec tion of C^{14} 4 pregnenolone System chloroform The Rf value obtained for C^{14} 4 pregnenolone was 0 04—0 08 in this system Compare Table 6 for Rf values of other reference substances used

	Blood				Brown fat		
	5		20		5		20
	♂	♀	♂	♀	♂	♀	♀
Rf 0 45—0 58	21	33	11	15	21	32	44
0 12—0 16	—	—	—	—	5	1	—
0 05—0 08	25	22	11	13	64	60	44
0 —0 05	54	45	78	72	10	7	12

DISTRIBUTION OF C^{14}-4-PROGESTERONE IN MICE

Progesterone is the most potent natural progestagen but also an intermediate in the biosynthesis of adrenocortical steroids and androgens and to some extent estrogens (cf Cook 1958) The physiology of progesterone is very complicated because it normally acts in conjugation with estrogens and it produces few specific changes when acting alone (cf Zarrow 1965) Labelled progesterone has been used to determine the concentration of the hormone in target sexual tissue by impulse counting both in rats and mice (Riegel et al 1950 Lawson and Pearlman 1964 Laumas and Farooq 1966) Autoradiography of the distribution of labelled progesterone in the uterus of rat was made by Rogers et al (1965) In order to compare the distribution pattern of progesterone in sites of synthesis of steroid hormones as well as in the whole body with the distribution pictures of cholesterol and pregnenolone a whole body autoradiographic study was undertaken

C^{14} 4 progesterone was injected to mice and after different survival times ranging from 2 minutes to 4 hours the animals were subjected to whole body autoradiography according to Ullberg Microautoradiography has also been carried out on some selected tissues

MATERIALS AND METHODS

C^{14} 4 progesterone was obtained from The Radiochemical Centre Amersham England The specific activity varied between 21 7 mci/mM (69 0 µci/mg) and 26 1 mci/mM (83 0 µci/mg) The radiochemical purity of the labelled progesterone was found to be > 97 % when tested with thin layer chromatography using cyclohexane ethylacetate (50 50) as a solvent

Twenty white mice (NMRI) were used for the experiments The progesterone was administered in different ways subcutaneously dissolved in arachidic oil and intravenously dissolved in ethyl alcohol or propylene glycol and water (50 50) or in an emulsion with Tween 80® The doses varied between 1 and 10 µci corresponding to about 0 01 and 0 14 mg progesterone

The intravenously injected animals (12 mice) were allowed to survive for 2 minutes (2 male mice and 4 female mice) 10 minutes (2 male mice) and 60 minutes (2 male mice 1 female mouse and 1 pregnant mouse) The eight subcutaneously injected mice were allowed to survive for 30 minutes (1 male mouse 1 female mouse and 1 pregnant mouse) 2 hours (1 male mouse 1 female mouse and 1 pregnant mouse) and 4 hours (1 male mouse and 1 fema

mouse) After the different survival times the animals were sacrificed and frozen and autoradiographed as described earlier (cf p 9)

From one male and one female mouse 2 minutes after i v injection and one male mouse 10 minutes after i v injection sections were also taken for microautoradiography as described before (p 10)

RESULTS

Very rapid excretion of the labelled progesterone was noted and very little radioactivity was seen in the body except in the bile and the liver after 4 hours The most interesting distribution patterns of C^{14} 4 progesterone were seen short time after intravenous injection No accumulation in the brain was observed after subcutaneous injection The blood concentration was very low 2 minutes after i v injection and the highest uptake was noted in the brown fat in the bile and in the bronchi Accumulation of radioactivity was also noted in the brain in the adrenal cortex and in the interstitial parts of the testes

The distribution in the various organs will be described in detail below

The circulatory system

The *blood* concentration was very low as soon as 2 minutes after i v injection and decreased gradually with time The *heart* showed a moderate uptake of C^{14} 4 progesterone 2 minutes after injection After 10 minutes and later the radioactivity in the heart was only slightly above that of the blood In the *lymphatic organs* spleen lymph nodes and thymus very weak concentration could be detected throughout the experiment

The adrenal gland

In the adrenal gland a high concentration could be seen in the cortex 2 minutes after intravenous injection of labelled progesterone (Fig 36) After 10 minutes the concentration in the adrenal cortex was rather low and only slightly above that of the adrenal medulla (Fig 43)

The reproductive system

Female

In the *ovaries* of non pregnant mice the highest uptake of C^{14} 4 progesterone was seen in the corpora lutea 2 minutes after injection (Fig 42) The concentration in the follicles was slightly higher than that in the interstitium In pregnant animals (late gestation state) the corpora lutea showed weak radioactivity slightly above the blood concentration In the non pregnant *uterus*

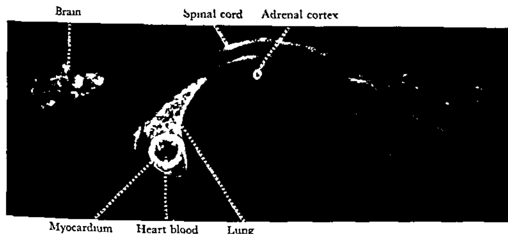


Fig 36 Autoradiogram of a female mouse 2 minutes after an i v injection of C 4 progesterone Note the high uptake (light areas) in the adrenal cortex The uptake in the brain also exceeds that of the circulating blood

and *vagina* very weak concentration could be noticed In some animals however the endometrium showed a little stronger uptake than the myometrium in the uterus

The *placenta* had a concentration corresponding to that of the blood The endometrium of the pregnant uterus showed slightly stronger radioactive concentration than the placenta The *fetuses* had rather low concentration The highest concentration was seen in the cartilage and the liver Intestinal contents contained slightly more C^{14} than the fetal organs

Male

The localization of C^{14} 4 progesterone and/or its metabolites was mainly confined to the interstitial cells of the *testes* (Fig 38) There was a high concentration 2 minu e after injection and this remained after 10 minutes In one animal an unev distribution was seen between different interstitial spaces some showing v y much higher uptake than the others In the *epididymis* different conce ations were seen Thus in the first segment(s) of the caput epididymidis t e was weaker accumulation of labelled progesterone than in an intermediate part where a distinct ring pattern of the tubules was seen (Fig 39) Ten minutes after injection the concentration in the epididymis was less tha in the interstitial parts of the testis but still showed the same distribution pattern within the epididym A strong uptake in *ductus deferens* was also seen (Fig 43) In the acce s ry sex glands rather low concentration was noticed except in the prost s which showed moderate concentration

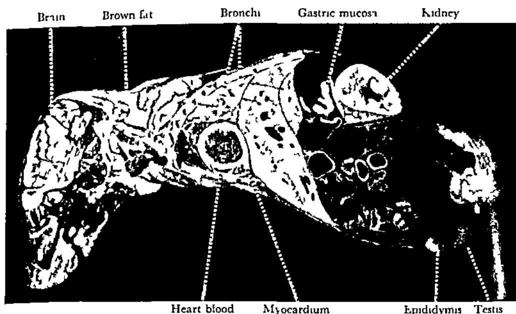
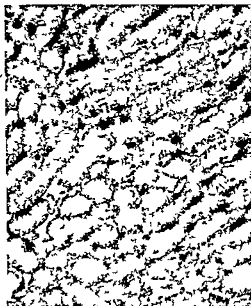
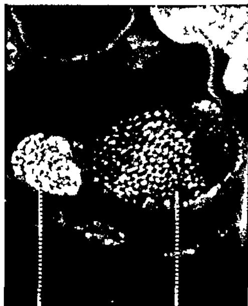


Fig 37 Autoradiogram of a male mouse 2 minutes after an i v injection of C'4 progesterone Note the high uptake (light areas) in brown fat brain and bronchi





Fi 38 Left Mic aut adiogram (section + autoradiogram) of a testis from a mouse 2 minutes after an i injection of C 4 progesterone The uptake (black grains) is very strong in the int it l ell Very little radioactivity was observed in the tubuli Htx eosin (x 26)

Fig 39 Right D l Fi 3 Note the high uptake (light areas) in part of the epididymis

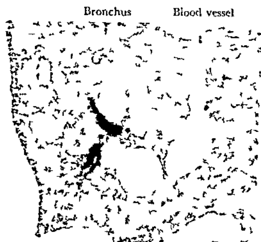


Fig 40 Microautoradiogram (section + autoradiogram) of the lung from a mouse 2 minutes after an i v injection of C^{14} 4 progesterone Very high uptake (*black grains*) can be seen in the bronchi No black grains can be seen in the blood Htx eosin (x 94)

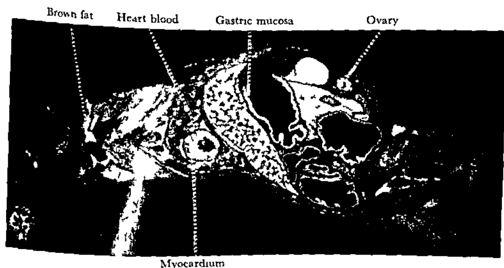
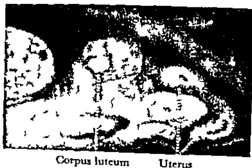


Fig 41 Upper Autoradiogram from a female mouse 2 minutes after an i v injection of C 4 progesterone Note the high uptake (light areas) in brown fat and ovary

Fig 42 Right Detail of Fig 41 Note the high concentration of C in the corpus luteum In uterus the endometrium seems to have a higher concentration than the myometrium



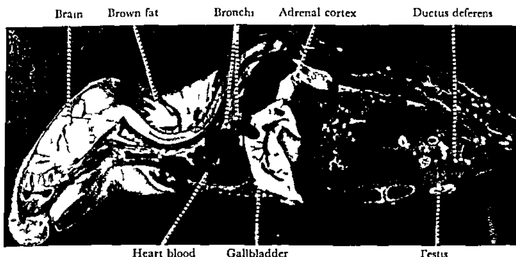


Fig 43 Autoradiogram of a male mouse 10 minutes after an i v injection of C^{14}-4 progesterone Note the high C^{14} concentration (light areas) in bile brown fat and ductus deferens The adrenal shows rather weak concentration

The nervous system

In the brain and spinal cord there was a strong accumulation of C^{14} 2 minutes after intravenous injection of C^{14}-4 progesterone There was higher concentration in the grey matter than in the white matter After 10 minutes the concentration in the brain had decreased considerably and most of the radioactivity was localized to the white matter Thirty minutes after injection the C^{14} contents in the central nervous system exceeded that of blood very little The *pituitary gland* showed moderate concentration and pars nervosa had slightly more radioactivity than pars distalis

The digestive system

No radioactivity could be detected in the *teeth* during the experiment The *salivary glands* showed the same concentration as the blood 60 minutes after intravenous injection As early as 2 minutes after injection a distinct line of radioactivity could be seen in the glandular part of the *gastric mucosa* After longer survival times a moderate concentration was seen in the stomach The *liver* showed a spotted pattern 2 minutes after injection As soon as 10 minutes after the injection the C^{14} concentration in the bile dominated the autoradiograms The liver had a rather heavy even blackening at that time Later on the concentration in the liver decreased but the bile still showed very high concentration of C^{14}

The respiratory system

A very high concentration could be seen in the *bronchi* from 2 minutes to 4 hours (Fig 40) The radioactivity seemed to be mainly localized to the epithelium The *lung parenchyma* showed a concentration which was slightly above that of the blood

The urinary organs

The kidneys showed a moderate concentration throughout the experiment There was rather high concentration of C^{14} in the urinary bladder 60 minutes after intravenous injection

The skin

One hour after subcutaneous injection there was a moderate uptake of radio active material in the hair follicles After 4 hours C^{14} could still be seen in the same places

Hard tissues

No radioactivity was seen in the *skeletal bone* In the *bone marrow* a low to moderate concentration of C^{14} 4 progesterone and/or its metabolites could be seen

The *skeletal muscles* did not accumulate any labelled progesterone during the experiment

The C^{14} 4 progesterone concentration in *brown fat* was very high 2 minutes after injection and remained rather high 30 minutes after injection There seemed to be slightly more C^{14} in the brown fat of the male mice than of the female mice The radioactivity decreased to slightly above the radioactivity in the blood after 60 minutes

DISCUSSION

It might be expected that progesterone would be localized in those tissues where it is known to exert a biological activity or in those tissues responsible for its inactivation P -gel et al (1950) using rats and mice registered low concentration of C in uterus and varying concentration in the ovaries while the pituitary anc orenal glands showed rather high C^{14} concentrations after injection of C^{14} l progesterone High uptake of H^3 in the brain 2 minutes after injection c l 2 H^3 progesterone was observed in rats by Laumas and Farooq (1966 a d they also noted a relatively low concentration of radio-activity in uteru and vagina

In the preser investigation usir g C^{14} 4 progesterone the highest C^{14}-concen trations in vari us organs w observed 2 minutes after i v injection F



Fig 44 Autoradiogram of brain from a rat 2 minutes after an i v injection of C' 4 progesterone Note the high uptake (light areas) in the grey matter The C concentration in the white substance is low (Appelgren unpublished)

concentration was noted in brown fat the bronchi brain adrenal cortex and interstitial parts of the testes There was a rapid decrease in all the organs mentioned and 4 hours after injection only the bile liver and intestinal contents were detectable in the autoradiograms indicating the rapid excretion of labelled progesterone described earlier by many investigators (for review see Fotherby 1964)

The uptake in the adrenals and in the reproductive system after injection of C^{14} 4 pregnenolone will be discussed in General discussion in comparison with the distribution of the other steroid hormone precursors studied

The nervous system

It is interesting to compare the uptake of labelled progesterone in the brain with some reported effects of progesterone in the central nervous system The feedback control of progesterone on anterior pituitary secretion seems to be exerted at the central nervous system level since intracerebral steroid implants are more effective than intrahypophysial implants (cf e g Sawyer 1966) Estrous behaviour is probably activated in hypothalamus according to both steroid implantation and lesion experiments (cf Sawyer 1960 Barraclough et al 1964) Progesterone has been reported to activate the estrous behaviour in ovariectomized rats after pretreatment with estrogen (Boling and Blandau 1939 Meyerson 1964)

The high uptake of tritiated progesterone in the brain and pituitary short time after injection observed by Laumas and Farooq (1966) was correlated to hypothalamo hypophysial interrelationsship They measured however the radioactivity in a portion of the brain that included not only a part of hypo thalamus but also adjoining cortical areas which probably contributed to the obtained values since the present autoradiographic findings in mice and

preliminary experiments in rats showed that the radioactivity was also to great extent found in the cortical areas (Fig 44)

The anaesthetic effect of progesterone reported by Selye (1941) and the use of progesterone derivatives as anaesthetics (cf Huguenard and Kern 1959) may also be correlated to the accumulation of labelled progesterone in the brain

Progesterone and its derivatives have been shown to have a prophylactic effect on migraine (Lundberg, 1962) The mode of action is not known but the high uptake of C^{14} progesterone in the central nervous system seen in the autoradiograms may indicate a central nervous effect rather than a direct action on blood vessels

The respiratory system

The pronounced uptake of labelled progesterone in the bronchi has been discussed earlier (p 51)

Skin

Progesterone has been reported to increase the size of the sebaceous glands in female spayed rats (Haskin et al 1953 van der Lely 1966) but in castrated male rats no such effect was observed (Ebling 1966) The uptake of labelled progesterone in the sebaceous glands was however similar in the autoradio grams from the male and female mice studied

Brown fat

The rapid elimination of injected progesterone from the blood in man is supposed to be due to an uptake in fatty tissue Accumulation was however seen only in brown fat in the autoradiograms There was a slightly higher uptake of labelled progesterone in the brown fat of the male mice than in that of the female mice This finding may be correlated to reports that brown fat probably secretes androgens (Sweet and Hoskins 1940 Pawlikowski 1955)



Fig 44 Autoradiogram of brain from a rat 2 minutes after an i v injection of C 4 progesterone Note the high uptake (light areas) in the grey matter The C' concen tration in the white substance is low (Ap pelgren unpublished)

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Impulse counting

0 1 ml of the chloroform extract and 0 1 ml of the water phase were counted in a liquid scintillation spectrometer (Packard) as described earlier (cf p 31) The tissue rests were measured with a GM counter (Tracerlab) after washing twice with line solution The % gram dose per gram tissue of the chloroform extracts was calculated

Chromatography

Chromatography was made on thin layer silica gel G plates according to Stahl (1962) and as described previously (cf p 36) After preliminary experi ment the following solvent systems were used for two dimensional chromato graphy

1 Methylene chloride acetone (80 20)
2 Chloroform ethylacetate (80 20)

In order to obtain R_f values of progesterone and some possible metabolites the following substances were used C^{14} 4 progesterone (The Radiochemical Centre Amer ham England) progesterone 17α OH progesterone 16 de hydroprogesterone dehydroepiandrosterone cortisone cortisol corticosterone deoxycorticosterone d aldosterone (Sigma Chemical Company Missouri USA) testosterone and estradiol (AB Leo Halsingborg) The R_f values obtained for the reference substances in the two dimensional system used are shown in Table 9 The chromatograms of the non radioactive substances were made visible by spraying the pl ites with o phosphoric acid (1 70) water (1 1) and heating them for 20 minutes at + 100 C and studied in ultra violet light The radioactivity of the chromatograms was detected with autoradiography as described earlier

RESULTS AND COMMENTS

The radioactivity in the washed tissue rests was less than 1 % of the combined counts in the w iter and chloroform phase The chloroform extracts from the different organs ere calculated as % gram dose per gram tissue and presented in diagrams (Fig 45)

These diag m do not represent the total amounts of radioactivity in the different org n since the water phase in some cases contained rather high concentration (C^{14} The water phase from the liver showed increasing values of C^{14} concen tion with time Two minutes ifter injection about 50 % of the total concen ation in the liver rem ined in the water phase and after 1 hour this figure h d increased to abo t 80 % Lawson and Pearlman (1964) found no progesterone in the liver af er injection of tritiated progesterone in rats

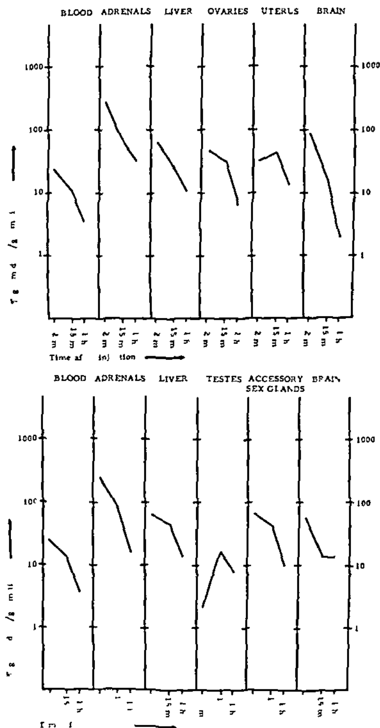


Fig 4. Radioactivity in chloroform extracts from various tissues of female mice (upper) and male mice (lower) at different intervals after i v injection of C 4 progesterone The rdinates give the contents per g m tissue as percentages of the dose given per gram

and stated that the rapid metabolic transformation to polar products was the reason In the other tissues studied there was not high enough concentration of C^{14} in the water phases for chromatography although rather high percentages of the total radioactivity were seen in some cases (Table 10) The radioactivity found in the water phases may represent conjugated progesterone

Adrenals

From the diagrams (Fig 45) can be seen that the adrenals have higher accumulation of chloroform extractable C^{14} per gram tissue than the other organs Two minutes after injection there are considerably more progesterone and/or its metabolites in the adrenals than in the blood Only 17 % to 34 % of the radioactivity in the chloroform extract from the adrenals at this time after injection had an R_f corresponding to reference progesterone

Testicles

The % gram dose/gram tissue values from the chloroform extracts of the testicles were rather low (Fig 45) However the concentration in the interstitial cells was considerably higher since the autoradiograms showed that practically all the radioactivity was localized to the interstitial tissue In the testicles only about 10 % of the radioactivity had an R_f corresponding to progesterone in the chloroform phase 2 minutes after injection and as much as about 30 % of the total radioactivity was found in the water phase At least 8 different radioactive spots were seen in the chromatograms One strong spot that might be testosterone according to the R_f value contained about 20 % of the radioactivity (Fig 46) Many more metabolites were seen in the chloroform extracts from the testicles 60 minutes after injection and at that time the bulk of the radioactivity was seen in the start and in spots with very low R_f values

Male accessory sex glands

About 37 % of the radioactivity in the chloroform extracts from the accessory sex glands corresponded to reference progesterone 2 minutes after injection The rest of the radioactivity was distributed amongst at least 10 metabolites Fifteen minutes after injection about the same amount of C^{14} was registered in the spot corresponding to reference progesterone as was registered after 2 minuts but the percental amount of radioactivity in the start and two spots with very low R_f values had increased to about 20 % from 7 % 2 minutes after injection

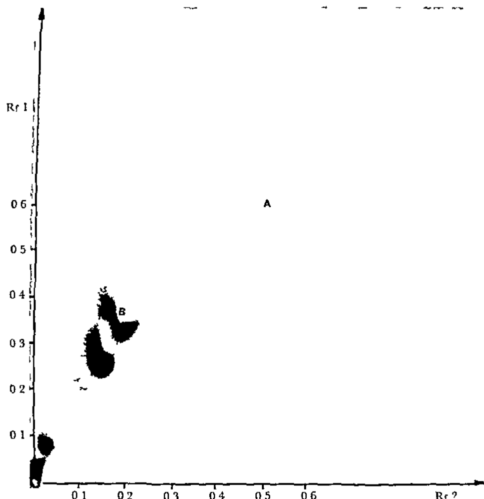


Fig 46 Two dimensional radiochromatogram of chloroform extract from testes of a mou e ? minutes after an i v injection of C 4 progesterone Very little radioactivity is seen with an R_f value corresponding to progesterone (A) The spot with R_f 0 35/0 1/ might correspond to testosterone (B)

Solvent system 1 Methylene chloride acetone (80 20)
2 Chloroform ethylacetate (80 ?0)

Ovaries

There was not sufficient radioactivity in the ovaries for chromatography although the concentration equalled that of uterus The autoradiograms revealed that some corpora lutea had considerably higher concentration than the rest of the tissue

Uterus

In the uterus only about 15 % of the radioactivity in the chloroform extract corresponded to the reference progesterone About 12 % was seen in a spot with an R_f value that agreed with that of estradiol *inter alia*

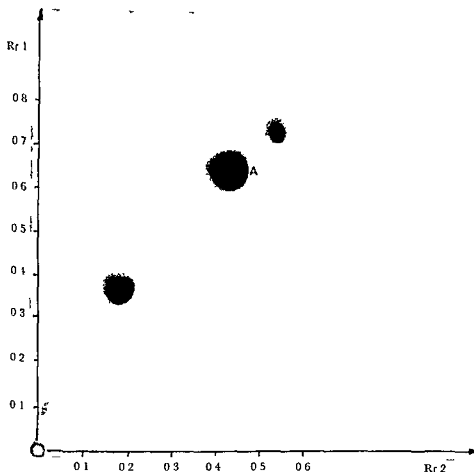


Fig 47 Two dimensional radiochromatogram of chloroform extract from a brain of a female mouse 2 minutes after an i v injection of C 4 progesterone About 65 % of the radioactivity has an R_f corresponding to progesterone (A)

Solvent system 1 Methylene chloride acetone (80 20)
Chloroform ethylacetate (80 ?0)

Brain

The radioactive concentration in the chloroform extract from the brain expressed as % gram dose/gram tissue was much higher than in the blood 2 minutes aft r injection of labelled progesterone and about 65 % had an R_f corresponding to progesterone in the chromatograms (Fig 47) Only 0 3 % of the radioa ivity remained in the water phase from the homogenized brain 2 minutes after injection Fifteen minutes after injection the concentration was only slightly higher than that of the blood But as seen from the autoradiograms most of the C^{14} was confined to the white matter and therefore the concentration in this part of the brain was considerably higher However only about 15 % of the radioactivity in the chloroform phase corresponded to reference progesterone at that t ne

Table 9 R_f values of reference substances obtained in the following two dimensional system

1 Methylene chloride — acetone (80 20)
2 Chloroform — ethylacetate (80 20)

	1	2
C^{14}-4-progesterone	0 60	0 44
progesterone	0 58	0 50
17 α-OH-progesterone	0 51	0 20
16-dehydroprogesterone	0 72	0 70
dehydroepiandrosterone	0 42	0 32
cortisone	0 19	0 01
cortisol	0 0[illegible]	0 03
corticosterone	0 12	0 06
deoxycorticosteron	0 35	0 22
d-aldosterone	0 09	0
testosterone	0 42	0 18
estradiol	0 39	0 27

Table 10 Per cent of total* radioactivity in the water phase in different tissues after various survival times after injection of C^{14} 4 progesterone

Time in min	Sex	Blood	Liver	Adrenals	Ovaries	Uterus	Brain
2	F	7	54	3	6	2	0 3
15		27	77	6	17	7	2
60		32	83	10	10	21	8
					Testes	Accessory sex glands	
2	M	3	47	3	28	0 1	1
15		32	79	12	10	10	4
60		—	74	30	18	18	10

* Total stands for the radioactivity in the combined chloroform and water extracts The small amount (< 1%) in the tissue rests was not considered

Conclusion

The chromatographic investigation gave information on the rapid metabolism and/or conjugation of C^{14} labelled progesterone There were also indications that other steroid hormones were formed since some of the radioactive spots had R_f values corresponding to reference steroid hormones

HISTOCHEMICAL LOCALIZATION OF Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE AND "SECONDARY ALCOHOL DEHYDROGENASE" ACTIVITY IN WHOLE BODY SECTIONS FROM MICE

The histochemical demonstration of hydroxysteroid dehydrogenases to indicate the sites of steroid production has been extensively used in the last ten years (for review see Baillie et al 1966) Wattenberg (1958) was the first to make histological localization of these enzymes possible He incubated unfixed tissue sections in a medium containing the hydroxysteroid to be investigated NAD a tetrazolium salt and buffer Hydrogen ions removed from the hydroxysteroid by the enzymes are transferred to NAD The reduced NAD is then transformed to NAD by reduced NAD tetrazolium reductase (diaphorase) while the tetrazolium salt is reduced to coloured formazan deposits Previous experiments using this technique have been made on isolated organs from different animals The use of whole body sections for incubation offers an interesting way for direct comparison of the localization of hydroxysteroid dehydrogenases in different organs in the body

Pathways from cholesterol to C_{21} derivatives involve scission of the side chain to form pregnenolone and isocaproic aldehyde or acid (Shimizu et al 1961) 20α hydroxycholesterol and 20α 22ξ dihydroxycholesterol are involved in this pathway from cholesterol to pregnenolone (Shimizu et al 1962) The enzymes involved in this pathway cannot be shown histochemically and Baillie et al (1966) claim that the presence of a cholesterol type side chain totally impedes histochemical investigation of 3β hydroxysteroids However Hardonk (1965) has shown the presence of a secondary alcohol dehydrogenase in human steroid producing cells and suggests that this enzyme might be involved with the oxidative cleavage of the cholesterol side chain He showed secondary alcohol dehydrogenase in the Leydig cells of the testis in the theca lutein cells in corpora lutea and in the zona reticularis of the adrenal in human biopsies Hardonk got the strongest histochemical reaction when using isopropanol as a substrate to demonstrate this secondary alcohol dehydrogenase In the present investigation incubations have been made with isopropanol using whole body mice sections to possibly indicate sites of cholesterol side chain scission

When pregnenolone is formed in or injected into the body it may be converted to progesterone by Δ^5 3β hydroxysteroid dehydrogenase This enzyme has been shown histochemically in many steroid producing cell systems in various animals using e g pregnenolone as a substrate In the present investigation histochemical experiments with pregnenolone or cholesterol as a substrate have been made with mouse whole body sections for direct comparison with the distribution of labelled pregnenolone in the autoradiograms

* NAD = nicotinamide adenine dinucleotide

MATERIALS AND METHODS

Whole body 10 μ thick freeze sections from NMRI mice were taken on tape 688 (Minnesota Mining and Manufacturing Co., USA) according to the method described earlier (p. 9). Two female mice, 1 male and 1 pregnant mouse (late gestation) were used. The sections were allowed to dry in the freeze room temperature (— 12° C). Otherwise the sections easily came off the tape during the incubation.

For demonstration of "*secondary alcohol dehydrogenase*" activity incubation with isopropanol was made according to Hardonk (1965) for 2 hours at + 37° C but omitting the acetone treatment prior to incubation. The incubation medium consisted of 0.5 ml isopropanol, 1.0 ml Nitro Blue Tetrazolium salt* (1 mg/ml), 0.5 ml nicotinamide adenine dinucleotide* (NAD) (5 mg/ml), 2 ml phosphate buffer pH 7.4 (0.2 M) and 2.3 ml distilled water. Adjacent control sections were incubated in the same medium but omitting the isopropanol or substituting it for ethanol.

For demonstration of *Δ-3β-hydroxysteroid dehydrogenase* incubation with pregnenolone* or cholesterol was made with the substance dissolved in dimethyl formamide (0.25 mg/ml) as suggested by Baillie et al. (1966). The same incubation medium as described above was used but replacing the isopropanol with 0.5 ml of the pregnenolone or cholesterol solution. When control sections were incubated the steroid solution was replaced by 0.5 ml dimethyl formamide. Some sections were briefly rinsed in acetone before incubation to dissolve possibly occurring endogenic hydroxysteroids.

As the last step in these enzyme reactions *is dependent on diaphorase some* incubations with reduced NAD were made to study the distribution of the diaphorase.

After incubating sections for 60 minutes to 2 hours they were briefly rinsed in phosphate buffer and fixed for 10 minutes in formalin (10 %) and embedded in glycerol gelatine.

RESULTS

"Secondary alcohol dehydrogenase

When isopropanol was used as a substrate no specific enzyme reactions were seen in adrenals, ovaries, testes and placenta. Formazan deposits were registered in Harder's gland, bronchi, liver and brown fat (Fig. 48, 49).

* Sigma Chemical Company, Missouri, USA.

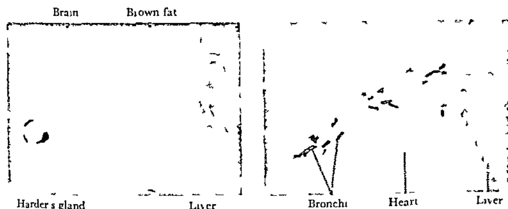


Fig 48 Left Detail of a whole body freeze section from a mouse showing the secondary alcohol dehydrogenase activity using isopropyl alcohol as a substrate Note the high enzyme activity (dark areas) in Harder s gland

Fig 49 Right Detail of a whole body freeze section from a mouse showing the secondary alcohol dehydrogenase activity (dark areas) in the bronchi

Δ^5 3β hydroxysteroid dehydrogenase

Pregnenolone

With pregnenolone as a substrate formazan deposits were seen in the adrenal cortex ovaries and testicles but also in some other tissues

Adrenal cortex The colour of the adrenal cortex was very strong and was evenly distributed through the whole cortex No clear differences between the sexes were seen (Fig 50 51)

Ovaries The strongest enzyme activity was registered in the corpora lutea from the pregnant mouse but enzyme activity was also registered in the interstitial tissue and some follicles In the ovaries from the nonpregnant mice the interstitial tissue had the strongest enzyme activity while corpora lutea and some follicles had less heavy formazon deposits Most of the precipitate in the follicles were confined to the theca cell layers (Fig 53 54)

Testes Weak Δ^5 3β hydroxysteroid dehydrogenase activity was observed in the interstitial parts of the testes (Fig 55) In the epididymis no enzyme activity was registered

Placenta Weak enzyme activity was observed in some cells in the decidua and yolk sac epithelium

Sebaceous glands Strong enzyme activity was seen both in the pregnenolone incubated and control sections In some sections rinsed briefly in acetone prior to incubation there was somewhat higher activity in the pregnenolone incubated sections than in the control sections (Fig 52)

Brain The brain showed weak Δ^5 3β hydroxysteroid dehydrogenase activity in the white matter (Fig 31 D)

In the *liver* and *brown fat* weak enzyme activity was also observed

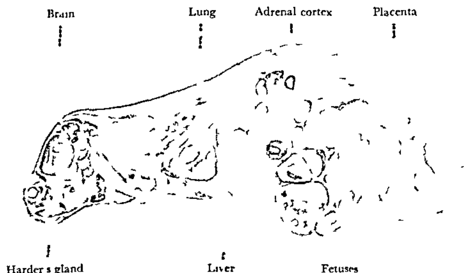


Fig 56 Whole body section from a pregnant mouse showing the distribution of diaphorase after incubation with reduced NAD Note the reaction in almost every tissue in the body Rather weak diaphorase activity was noted in white matter in the brain

Cholesterol

When cholesterol was used as a substrate no formazan deposits were registered that did not appear in the control sections

Diaphorase

The incubation with reduced NAD gave heavy deposits of formazan in almost every tissue in the body (Fig 56) The strongest reaction in the body was seen in the sebaceous glands Rather weak diaphorase activity was noted in the white matter of the brain and in the granulosa cells of the ovary

DISCUSSION

The advantage of using whole body sections in histochemical localization of dehydrogenase activities is that the relative concentration of enzyme activity in different tissues treated in exactly the same way can be compared in the same section

The demonstration of dehydrogenase depends on the transfer of hydrogen removed from the hyd ... via a pyridine nucleotide to a tetrazoleum salt which is reduced ... ited at the site of reaction This last step is dependent on the pres... phorase Therefore control incubations with reduced NAD were m... ative results in some cell systems were probably not due to lack ... as was seen from the incubations with reduced NAD

Secondary alcohol dehydrogenase

In this investigation "secondary alcohol dehydrogenase activity could not be established in mice in the same places as has been reported in humans (Hardonk 1965) Hardonk found very strong enzyme activity in the Leydig cells of the testis in the theca cells of the ovary and in the zona reticularis of the adrenal in human biopsies None of these cell systems showed any enzyme activity in the present investigation The only tissues where this enzyme activity was demonstrated in mice were Harder s gland bronchi liver and brown fat The enzyme activity in these tissues can probably not be correlated to the cholesterol side chain cleavage The present autoradiographic investigation of labelled cholesterol showed that cholesterol accumulated in the liver but very little C^{14} was seen in Harder s gland and brown fat and none in the bronchi Thus these findings in mouse do not support Hardonk s theory that the secondary alcohol dehydrogenase may be identical with the enzyme that catalyses the cleavage of the cholesterol side chain

Δ -3β hydroxysteroid dehydrogenase

The high selective Δ^5 3β hydroxysteroid dehydrogenase activity in the adrenal cortex and in the ovary is well illustrated by the incubated whole body sections The enzyme activity in other tissues was difficult to detect without magnification in a microscope

In the *adrenal cortex* Wattenberg (1958) found that all cells had enzyme activity when using pregnenolone as a substrate but the reaction in zona glomerulosa was weak In a study of the developing mouse adrenal Allen (1960) found the same but also pointed out that there was enzyme activity in the female X zone while it was missing from the male X zone The present investigation showed that the Δ^5 3β hydroxysteroid dehydrogenase activity was present in the whole cortex in both male and female mice

In mouse *ovaries* Δ^5 3β hydroxysteroid dehydrogenase has been shown with pregnenolone (Ferguson 1965) and dehydroepiandrosterone (Deane and Rubin 1965) as substrates Their findings agree with the present investigation Enzyme activity was seen in corpora lutea interstitial tissue and to less extent in theca interna and some granulosa cells The granulosa cells showing enzyme activity are supposed to be atretic by Deane and Rubin (1965) The enzyme reaction in the granulosa cells may be limited by the low diaphorase concentration there noted in the present investigation and previously observed by Baillie et al (1966)

Δ^5 3β hydroxysteroid dehydrogenase activity has been reported in the trophoblastic giant cells in the rodent *placenta* (Deane et al 1962 Botte et al 1966) These authors found the maximum enzyme activity at 15 days after mating in the mouse The weak enzyme activity seen in this investigation may be due

In the Leydig cells from mouse *testes* Δ 3β hydroxysteroid dehydrogenase activity using pregnenolone as a substrate has been found (Hitzeman 1962 Baillie and Griffiths 1965) Hitzeman found a much weaker reaction when pregnenolone was used instead of dehydroepiandrosterone Weak enzyme activity in the interstitial cells was also seen in the present experiments

Δ^5 3β hydroxysteroid dehydrogenase activity has been shown using pregnenolone as a substrate in *sebaceous glands* from human biopsies (Baillie et al 1965) but not from mouse (Baillie 1966) In the present investigation however enzyme activity was demonstrated in mouse sebaceous glands in sections that were rinsed in acetone prior to incubation The strong reaction seen in the control sections not rinsed in acetone may be correlated to the abundant concentration of diaphorase in the sebaceous glands seen in this investigation Even small amounts of endogenous hydroxysteroids might therefore give strong formazan reaction

The present histochemical results will be further discussed when comparing the autoradiographic distribution of the labelled precursors in General discussion

GENERAL DISCUSSION

The autoradiographic distribution of the different steroid hormone precursors in endocrinologically active tissues will be discussed together with some histochemical and biochemical evidence for steroid hormone formation found in other laboratories To some extent other organs than endocrine will also be discussed

THE ADRENAL CORTEX

The principal adrenocortical steroid hormones i e cortisol corticosterone and aldosterone are found in a wide variety of vertebrate species and may well be present in all of them (cf Bush 1962) Whether androgens estrogens and progesterone are secreted by the adrenal cortex of all mammals is still a matter of debate

There are data in the literature indicating species differences Triller and Birmingham (1965a b) have investigated the steroid production by incubated mouse adrenals and have found that the main products were corticosterone and aldosterone No evidence for the formation of 17-hydroxylated steroids (e g cortisol and cortisone) was found nor did they find 18 hydroxy-11 deoxy corticosterone a major secretory product of the rat adrenal (Vinson 1966) However Karaboyas and Koritz (1965) have been able to demonstrate 17α hydroxylase activity in rat adrenal cortex *in vitro* Although species differences concerning the final steroids produced by the adrenal cortex have been indi cated the initial steps from cholesterol to pregnenolone and progesterone seem to be common for all species studied (cf e g Caspi 1961 Dorfman 1961 Grant 1962)

There are somewhat conflicting theories regarding the role of different zones of the adrenal cortex Some authors claim that each zone is independent in hormone production while others suggest an interrelation

Swann (1940) suggested that there may be a division of secretory function between the zones of the adrenal cortex These functions may not be equally dependent on the hypophysis After hypophysectomy the zona glomerulosa remained unchanged for some time while the zona fasciculata rapidly atro phied

Later direct demonstration of s roid production within the histologically defined zones in the adrenal c rtex has been performed by Ayres et al (see Ayres 1960) They found th in ox adrenal the 11β hydroxylase for corti

costerone production was present in both the zona glomerulosa and zona fasciculata while the 17α hydroxylating system for cortisol production was confined to the zona fasciculata and the 18-oxidase for aldosterone production was confined to the zona glomerulosa According to Grant (1962) 11β hydroxylase and 21-hydroxylase (for e g deoxycorticosterone production) are located in all zones and 18 hydroxylase in the zona glomerulosa only and 17α hydroxylase is present in both zona fasciculata and zona reticularis Δ^5-3β hydroxysteroid dehydrogenase for transformation of e g pregnenolone to progesterone has been demonstrated in the whole mouse adrenal cortex in the present investigation (cf Wattenberg 1958 Allen 1960)

C[14] cholesterol

No information on the site of side chain cleavage and/or the hydroxylation of cholesterol in 20- and 22 position within the different zones in the adrenal cortex is yet available Therefore it is interesting to notify the specific accumulation of labelled cholesterol in zona fasciculata seen in the autoradiograms A slight uptake was also seen in zona glomerulosa Symington (1962) has proposed that zona fasciculata represents a storage zone of steroid precursors mainly sterol esters and this is in agreement with the present investigation which has shown that after 4 days the accumulated radioactivity in the adrenal represents mainly cholesterol esters (p 40) Davis and Garren (1966) have indicated that adrenocorticotropic hormone activates the conversion of cholesterol esters to free cholesterol as a first step in the synthesis of steroid hormones

There were great differences in the kinetics between exogenous cholesterol and pregnenolone and progesterone in the adrenal cortex Cholesterol was taken up very slowly in the adrenal cortex It did not reach its maximum concentration until 1 to 4 days after injection and then it was slowly decreasing Pregnenolone and progesterone reached their maximum concentrations in the adrenals 2—5 minutes after injection The concentration of these two latter hormone precursors then decreased rather rapidly The reason for this difference between cholesterol on one hand and pregnenolone and progesterone on the other is probably explained by a more rapid utilisation of the latter ones in the synthesis of cortical hormones These observations *in vivo* support the view from *in vitro* experiments that conversion of cholesterol to pregnenolone includes the slow step(s) in steroid synthesis (Ichii et al 1963 Koritz and Hall 1964 Hall and Koritz 1964 Ichii et al 1965)

C[14] pregnenolone

As mentioned above the highest concentration in the adrenal cortex was seen 5 minutes after injection i C[14] 4 pregnenolone The radioactivity then

decreased rather rapidly probably due to steroid hormone formation The radioactivity in the adrenal cortex 5 minutes after injection probably represented pregnenolone only to a small extent and most of the radioactivity represented more polar steroids possibly corticosteroids as indicated by the present chromatographic investigation The distribution within the cortex 5 and 20 minutes after injection of labelled pregnenolone was very even and thus in accordance with the Δ 3β hydroxysteroid dehydrogenase distribution when pregnenolone was used as a substrate This shows that the conditions for the formation of Δ^4-3β ketosteroids (e g progesterone) are present in the whole adrenal cortex in mice While the C^{14} of the labelled progesterone had totally disappeared after 4 hours small amounts of C^{14} evenly distributed in the adrenal cortex could be detected 24 hours after injection of C^{14} 4 pregnenolone It has been suggested that pregnenolone has a regulatory effect on steroid hormone formation because of its inhibitory action on the transformation of labelled cholesterol to 20α hydroxycholesterol *in vitro* (Koritz and Hall 1964) It is therefore possible that there always is some pregnenolone in the cortex and that the weak radioactivity registered there after 24 hours might represent non metabolized pregnenolone

C^{14} progesterone

The rapid disappearance of C^{14} 4 progesterone from the adrenal cortex indicates a rapid transformation to steroid hormones and release of the products formed The adrenocortical hormones are thus probably released in rather close connection to their formation

Vinson (1966) has suggested from *in vitro* studies with H^3 pregnenolone and C^{14} progesterone with rat adrenals that the conversion of radioactive progesterone into corticosteroids is more rapid than that of pregnenolone He found that maximal incorporation of isotope from C^{14} progesterone into corticosteroids occurred earlier than maximal incorporation from H^3 pregnenolone

In the present experiments the occurrence of several metabolites were demonstrated 2 minutes after injection of labelled progesterone and more than 50% of the radioactivity represented more polar compounds than progesterone No differences in the distribution among the different zones in the adrenal cortex was observed in the autoradiograms and this finding may be compared with the earlier mentioned distribution in all cortical zones of 11β and 21 hydroxylase needed for e g transformation of progesterone to deoxycorticosterone and corticosterone

THE OVARY

Corpora lutea

Among the different structural subunits which are endocrinologically active in the ovary the corpus luteum is generally accepted as the source of ovarian progesterone Labelled cholesterol has been shown to be used as precursor to progesterone with pregnenolone as an intermediate when incubated *in vitro* with preparations from bovine corpus luteum (Tamaoki and Pincus 1961 Ichii et al 1963 Hall and Koritz 1964 Mason and Savard 1964)

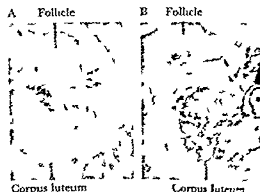
C^{14} cholesterol

In this investigation labelled cholesterol was shown to accumulate in corpora lutea and thus probably serves as a precursor for the steroid hormones formed there Chromatographic investigation showed that 4 hours after injection about 10 % of the cholesterol in the whole ovary was in esterified form but after 10 and 20 days about 90 % of the C^{14} represented esterified cholesterol No metabolites which indicated the formation of steroid hormones could be registered This may be due to a very slow formation of steroid hormones from cholesterol which is supported by the fact that labelled cholesterol was taken up slowly and that the C^{14} concentration remained fairly high during very long time in the ovaries Labelled pregnenolone and progesterone on the other hand reached their maximum concentration 5 minutes after injection followed by a rather rapid decrease in concentration Progesterone seemed to disappear more rapidly than pregnenolone which fits into the general pattern of steroid hormone formation

C^{14}-pregnenolone

Also C^{14} 4 pregnenolone has been shown to accumulate in the corpora lutea in this investigation The specific accumulation of C^{14} 4 pregnenolone and/or its metabolites is in agreement with the Δ^5-3β hydroxysteroid dehydrogenase activity seen in the corpora lutea of pregnant mice when pregnenolone was used as a substrate in this investigation (cf Rubin et al 1963 Deane and Rubin 1965)

Very pronounced accumulation in corpora lutea was also found in autoradio graphic experiments with a non steroid anti fertility compound bis (p hydroxy phenyl) cyclo hexylidenemethane acetate F6066 shown by Hann gren et al (1965) The accumulation of F6066 in corpora lutea may be con nected with the progesterone formation This possibility is supported by *in vitro* experiments (Larsson and Stenson 1967) with homogenates of ovaries where the transformation of pregne lone to progesterone was studied This reaction



Fi 5i Details of ovaries from whole body sections from a pregnant mouse showing the Δ^5 3β hydroxysteroid dehydrogenase activity using pregnenolone as a substrate A shows the inhibitory effect on enzyme activity of F6103 added to the incubation medium B is the corresponding control section (Appelgren unpublished)

appeared to be inhibited by F6060 and these authors postulated that [illegible] hydroxysteroid dehydrogenase and/or isomerase is/are inhibited A [illegible] compound F6103 has been shown to decrease the 3β hydroxy [illegible] hydrogenase activity in corpora lutea with histochemical methods [illegible] enolone was used as a substrate (Appelgren unpublished) (Fig [illegible]) [illegible] pregnenolone as well as the Δ^5 3β hydroxysteroid dehyd[illegible] was found to be rather selectively localized in the corpora [illegible] that pregnenolone as a precursor for steroid hormones is main[illegible] lutea and only to a small extent in other ovarian structures (cf [illegible]

C^{14} progesterone

Labelled progesterone was not expected to appear in the [illegible] this should indicate that the hormone is specifically [illegible] where it is produced (cf Ullberg and Bengtsson 1963) [illegible] after injection an accumulation of labelled progesteron[illegible] was noted in the corpora lutea of a non pregnant mou[illegible]

However on a comparative biochemical basis tw[illegible] seem to exist The corpora lutea from cow and mare [illegible] only to produce progestins while those from man [illegible] steroids including estrogens (Savard et al 1965) [illegible] for mouse in this respect but it is tempting to pre[illegible] belongs to the same group as the rat ovary [illegible] corpora lutea therefore may act as a precursor [illegible] e g androsterone (Simmer and Voss 1960) As [illegible] taking place in the corpus luteum can be mentio[illegible] was taken up in corpora lutea (App[illegible]lgren unpu[illegible] bilities for further metabolism of te[illegible]tosterone [illegible] (1959 who using testosterone as a substrate de[illegible] dehydrogenase activity in rat cor[illegible]ra lutea

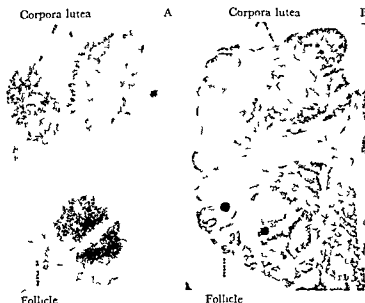


Fig. 58 A Detail of whole body autoradiogram showing the distribution of C^{14} in the ovary from a pregnant mouse 24 hours after an i v injection of C^{14} 4 pregnenolone Note the high uptake (black areas) in the corpora lutea

B Detail of the corresponding section to A showing the Δ^5 3β hydroxysteroid dehydrogenase activity using pregnenolone as a substrate Note the enzyme activity in the corpora lutea

Balogh (1964) suggested that 20α hydroxysteroid dehydrogenase activity was an indicator for progesterone metabolism and showed that such activity was great in rat corpora lutea in involution Thus the uptake in some corpora lutea after injection of C^{14} 4 progesterone might indicate metabolism of progesterone and its 20α hydroxysteroid derivatives

Ovarian structures other than corpora lutea

There has been divergence of opinion about the site of production of estrogens in the ovaries In early histochemical literature (for review see e g Falck 1959) the theca interna cells were postulated to be active in hormone production while the granulosa cells played little if any role Claesson et al (Claesson and Hillarp 1947 a b Claesson et al 1948 1953 Aldman et al 1949 Claesson 1954) extensively studied the lipids mainly the cholesterol fraction in the theca interna and interstitial gland and found a variation in the chole terol content of these cells that could be correlated to the secretion of estrogens Falck (1959) suggested that both the granulosa and theca cell layers were necessary for estrogen biosynthesis Recent investigations however suggest that the granulosa cells alone have the capacity for aromatization as well as other steroid transformation (Bjersing and Carstensen 1964 Ryan and Short 1965)

C^{14} cholesterol

The present investigation with labelled cholesterol supports the findings of Claesson et al (see above) concerning the distribution of cholesterol in the

ovary but radioactivity was not only found in the interstitial gland and theca interna but in a low concentration also in the granulosa cell layers and even in the ova These findings might be due to higher sensitivity of the method used or to the formation of labelled metabolites

The variations in steroid content of the interstitial gland in the rat ovary during the estrous cycle seen by Claesson and Hillarp (1947b) were not noted in mice when the localization of labelled cholesterol was studied at different intervals after injection Chromatographic experiments indicated a slow esterification of the labelled cholesterol in the ovaries This may explain the differences between this investigation and that of Claesson and Hillarp (1947b) Claesson et al (1948) observed that the concentration of esterified cholesterol varied much more than that of free cholesterol

When estrogenic hormones are formed from injected C^{14} 4 cholesterol they may be expected to localize in target organs e g the granulosa cell layers of the follicles as shown by Ullberg and Bengtsson (1963) When C^{14}-26 cholesterol is used the steroid hormones formed are non labelled and therefore cannot cause any autoradiographic blackening But probably due to the slow production of hormones no significant differences in the distribution in the granulosa cell layers could be noted when C^{14} 4 cholesterol or C^{14} 26 cholesterol was injected

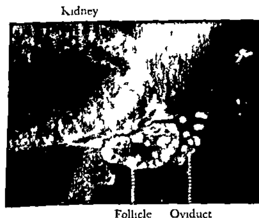
A possibility which may at least theoretically be considered is that the C^{14} labelled isocaproic acid split off from the side chain labelled cholesterol localized in the granulosa cell layers (cf Fig 59 Appelgren unpublished)

Bjersing and Carstensen (1964) and Ryan and Short (1965) have shown the ability of granulosa cells in some species to transform steroid hormones *in vitro* using e g pregnenolone and testosterone as precursors The present findings may indicate a precursor role also for cholesterol there

The radioactivity in the ova after injection of cholesterol may be correlated to the finding of some hydroxysteroid dehydrogenases in mouse ova (Baillie et al 1966)

C^{14} pregnenolone

Labelled pregnenolone and/or its metabolites did not accumulate to the same extent in the interstitium and follicles as in the corpora lutea which may indicate that pregnenolone either was transformed and given off rapidly or was not used as a precursor there However isolated granulosa cells from pig have the ability to transform pregnenolone to progesterone (Bjersing and Carstensen 1964) Δ^5 3β hydroxysteroid dehydrogenase activity using pregnenolon as a substrate was rather strong in the interstitium but modest in most follicle walls Some of the large follicles possibly atretic ones showed higher enzyme activity in their walls than others According to Deane and R



Fi 59 Autoradiogram of an ovary from a mouse 24 hours after an i v injection of C 1 isocaproic acid Note the high uptake (light areas) in the follicles and oviduct (Appelgren unpublished)

(1965) atretic follicles showed higher enzyme activity than other follicles when incubated with dehydroepiandrosterone as a substrate

C^{14} progesterone

Labelled progesterone was not taken up to any great extent in the ovaries except in the corpora lutea as described above Some radioactivity however could be detected in some follicle walls short time after injection but dis appeared very rapidly As was previously mentioned Balogh (1964) used the 20α hydroxysteroid dehydrogenase activity to indicate progesterone metabolism and he found that in rat also atretic follicles showed this enzyme activity It is interesting to compare his finding with the uptake of radioactivity seen in some follicles possibly atretic in the present investigation

UTERUS

The uterine wall especially the endometrial glands was shown to accumulate estrogens by Ullberg and Bengtsson (1963) and thus may be supposed to be specifically taking up estrogens formed from the injected labelled precursors

C^{14} cholesterol

No differences in uptake in uterus was seen after injection of the two dif ferently labelled cholesterol compounds indicating that cholesterol as such was taken up there making it impossible to detect small amounts of estrogens if formed

^{14} pregnenolone

Pregnenolone and/or its metabolites was not taken up in uterus to any great ent which might indicate poor formation of estrogens from this precursor

C^{14} progesterone

The effects of progesterone on uterus might be assumed to be accompanied by the absorption of C^{14} 4 progesterone but radioactivity measurements have shown very low concentration (Riegel et al 1950 Laumas and Farooq 1966) This agrees with the present investigation There was however slightly higher concentration of C^{14} in the endometrium than in the rest of the uterus after injection of C^{14} 4 progesterone Rogers et al (1965) found the highest concentration of radioactivity in the myometrium 6 hours after injection of C^{14} 4 progesterone or generally labelled H^3 progesterone in pseudopregnant ovariectomized rats and in the stroma between the myometrium and endometrium when the uterus horn was traumatised The present chromatographic experiments have shown a metabolite that might be estradiol in the uterus 15 minutes after injection of C^{14} 4-progesterone If this is true the radioactivity in the endometrium may be due to formed estrogens since they have been shown to localize in the endometrial glands (Ullberg and Bengtsson 1963)

Thus the difference in distribution may be due to formed estrogens from C^{14} 4 progesterone in the ovary in the present investigation since Rogers et al used ovariectomized rats

PLACENTA

Progesterone is essential for the maintenance of pregnancy and it is generally accepted that the human placenta takes part in the formation of this hormone The placenta appears to be an incomplete endocrine organ depending upon extra placental steroid precursors formed by the mother and by the fetus (cf e g Diczfalusy et al 1965 Ryan et al 1966) The role of the rodent placenta in production of steroid hormones is debated and ovariectomy causes abortion in rat and mouse There are however both biochemical (Harkness et al 1964 Ratsimamanga et al 1964 Vinson and Chester Jones 1964 a) and histochemical evidence for metabolism and secretion of steroid hormones in the rodent placenta The trophoblastic giant cells are thought to be responsible for the steroid hormone production (Deane et al 1962 Botte et al 1966)

Of the injected precursors only labelled *pregnenolone* showed a specific localization in the mouse placenta in late gestation state in the autoradiograms C^{14} 4 pregnenolone and/or its metabolites localized around some blood lacunae adjacent to decidua basalis probably in the giant trophoblast cells 5 minutes after injection This localization agrees with that found for Δ^5-3β hydroxy steroid dehydrogenase in the trophoblast giant cells in rat placenta with DHA (Deane et al 1962) or pregnenolone (Botte et al 1966) as a substrate After longer survival times labelled pregnenolone and/or its metabolites were localized mostly in the yolk sac epithelium Weak Δ^5 3β hydroxysteroid dehydrogenase activity was noted in this place in the whole body sections incubated

with pregnenolone in the present investigation Botte et al (1966) found 17β hydroxysteroid dehydrogenase which is specific for 17β hydroxysteroids with an aromatic A ring e g estradiol 17β in the yolk sac villi of the rat placenta

After injection of labelled *cholesterol* there was some radioactivity evenly distributed in the labyrinth and somewhat higher C^{14} concentration in the yolk sac If there is a specific localization of cholesterol within the placenta the survival period (60 minutes) may be too short for its detection since cholesterol accumulation has been shown to be very slow in other sites of steroid hormone synthesis

Only small quantities of evenly distributed radioactivity was seen in the mouse placenta after injection of labelled *progesterone* and this is in agreement with the presumption that a hormone is not taken up in a tissue in which it is produced

THE FETUSES

Although rodent fetal tissues are known to take part in steroid transformation *in vitro* (Vinson and Chester Jones 1964a) only small amounts of the injected labelled precursors were found in the fetuses in the present investigation

MALE SEX ORGANS

Androgens are essential for the complete and normal function of the seminiferous tubules in mice and other species as well (cf Turner 1960) The accessory system of male ducts and glands are morphologically and physiologically dependent upon the production of androgens Labelled testosterone injected to mice would therefore very likely be localized in both the seminiferous tubules and accessory sex glands However preliminary investigations on the localization of C^{14} 4 testosterone in mice after intravenous injection (Fig 60) show that there was rather high C^{14} concentration in the interstitial cells and very low level of radioactivity in the seminiferous tubules of the testes High accumulation of testosterone and/or its metabolites was seen in ductus deferens and moderate accumulation in epididymis The accessory sex glands showed only low concentration of radioactivity If the injected precursors are to any considerable extent transformed into testosterone the labelled products are likely to appear in ductus deferens and in epididymis after some latency in the autoradiograms Radioactivity could be found in ductus deferens after injection of ring labelled cholesterol progesterone and pregnenolone but also after injection of side chain labelled cholesterol which indicates that at least in the case of cholesterol the precursor itself was localized there The epididymis had a high concentration of C^{14} very shortly after injection of C^{14} 4 progesterone which probably cannot represent synthesized testosterone

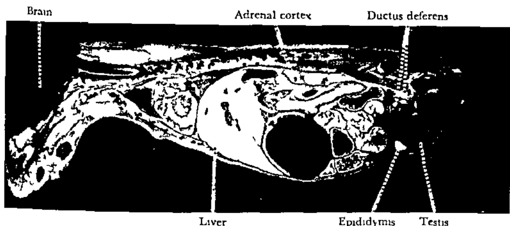


Fig 60 Autoradiogram of a male mouse 5 minutes after an i v injection of C 4 testo terone Note the uptake (light areas) in the adrenal cortex ductus deferens epididymis and interstitial cells in the testis (Appelgren unpublished)

The main source of androgens in the testes of laboratory mammals is the interstitial or Leydig cell The Δ^5-3β hydroxysteroid dehydrogenase histo chemical reaction localizing a step in androgen biosynthesis is positive in the interstitial cells but negligible in the seminiferous tubules of several species (Wattenberg 1958 Levy et al 1959 see also p 79) The pathway for biosynthesis of testicular androgens in rat is similar to that found in adrenal and includes the formation of testosterone and androstenedione by way of cholesterol pregnenolone and progesterone (cf Hechter 1958) Some *in vitro* experiments in mice have shown a more rapid utilization of tritiated pregn enolone than C^{14} progesterone in the formation of androgens in mouse testis (Ellis and Berliner 1965)

In the present investigation it has been shown that exogenous cholesterol is taken up and stored to a great extent in the interstitial tissue of the testicle in mouse indicating the possibility for cholesterol to be precursor for steroid hormone synthesis Labelled pregnenolone and progesterone were also shown to be localized in the interstitial parts of the testicles which supports the postulated pathway of androgen biosynthesis from cholesterol via pregn enolone and progesterone That the radioactivity disappears rather rapidly from the testicles after injection of C^{14} 4 pregnenolone and C^{14} 4 progesterone may be due to formation of androgens or other metabolites that are not stored very long

The concentration of C^{14} in the test ics increases with time after injection of both C^{14} 4 cholesterol and C^{14} 26 holesterol up to at least ten days which may demonstrate the slow step in roid hormone transformation mentioned earlier Dorfman et al (1967) su ested after *in vitro* studies with rat testicular

tissue that the 20α hydroxylation of cholesterol is the rate limiting step in the biosynthesis of pregnenolone from cholesterol

Christensen and Fawcett (1966) have suggested that the extensive membranes of the agranular reticulum which are found in mouse interstitial cells in addition to providing sites for enzymes may also act as a reservoir for the storage of cholesterol

In mice Δ^5-3β hydroxysteroid dehydrogenase has been demonstrated in the seminiferous tubules when using dehydroepiandrosterone sulphate as a substrate (Baillie and Griffiths 1965) In a biochemical study in rat by Christensen and Mason (1965) it has been shown that although the interstitial tissue is the predominant source of androgens the seminiferous tubules are also capable of some production *in vitro* Woods and Domm (1966) using a modified fluorescent antibody technique also found that seminiferous tubules of chicken and to less extent those of the rat were capable of androgen synthesis Minute amounts of radioactivity could also be demonstrated in the seminiferous tubules after injection of labelled cholesterol pregnenolone and progesterone in the autoradiograms a finding that might support the above mentioned suggested ability of the seminiferous tubules to produce androgens The radioactivity in the seminiferous tubules might be due to the formation of labelled steroid hormones but this does not seem likely since the distribution of side chain labelled cholesterol was similar

The radioactivity in the epididymis after injection of C^{14} 4 progesterone was not evenly distributed There was a high uptake in part of caput epididymidis and this uptake was confined to the epithelium This localization within the epididymis is interesting to compare with the variation of different hydroxysteroid dehydrogenases in different zones in the epididymis (Baillie et al 1966)

THE NERVOUS SYSTEM

Naturally occurring steroids are known to exert influence on central nervous functions such as behaviour motion sleep wakefulness and excitability In high dosage various steroids may act as anaesthetic anticonvulsive or epileptogenic drugs (cf Hamburg 1966 Sawyer 1966) These "pharmacological effects are not necessarily dependent on the physiological or hormonal properties of the steroids (Craig 1966)

Cholesterol high doses has been reported to induce anaesthesia by Cashin and Moravek (?7) Selye (1941) however found no anaesthetic effect of cholesterol in do s up to 500 m /c in rats The autoradiograms showed that the penetrati into the brain belled cholesterol was very slow No acute effects on the ntral nervous sys ay therefore be expected

Labelled progesterone and pregnenolone on the other hand accumulated in the brain very short time after injection The concentration of both these substances was very high in the grey matter and low in the white matter short time after injection With time the pattern in the brain changed and the concentration was highest in the white matter 10 and 20 minutes after injection of labelled progesterone and pregnenolone respectively

The finding of labelled *progesterone* in the central nervous system may be explained by its hormonal actions such as the feedback control on anterior pituitary secretion (cf Laumas and Farooq 1966) and the ability of progesterone to activate estrous behaviour (Boling and Blandau 1939 Meyerson 1964)

However the present autoradiographic findings may also be correlated to some centrally depressing effects of progesterone Selye (1941) reported an anaesthetic effect of progesterone and later a progesterone derivative (Viadril®) has been used as an anaesthetic drug (cf Huguenard and Kern 1959) Progesterone in subanaesthetic doses facilitates the occurrence of natural sleep (Heuser 1967) Prophylactic effect on migraine of progesterone and some derivatives have been reported by Lundberg (1962)

No hormonal actions on the central nervous system have been found for *pregnenolone* but some depressing effects which are reported may be compared with the uptake of labelled pregnenolone in the brain The anaesthetic effect of pregnenolone was very slight in rats although high doses were given (500 mg/kg) (Selye 1941) An anticonvulsive action of pregnenolone has been reported by Spiegel (1946 1950) in cats In mice however this effect was negligible (Spiegel and Wycis 1945 Craig 1966)

SUMMARY

Radioactively labelled steroid hormone precursors were found to be taken up specifically in tissues engaged in steroid hormone synthesis

Autoradiographic localization of the precursors therefore was used to obtain information on the sites of synthesis of steroid hormones on a tissue and cell level The precursors used were C^{14} labelled cholesterol pregnenolone and progesterone

The kinetics in the biosynthesis chain was elucidated by following the uptake and disappearance rate of the labelled substances The autoradiographic data were supplemented with values obtained by impulse counting

The chemical identity of the labelled substances was followed by radio chromatography Interpretation of the cholesterol autoradiograms from a chemical identity viewpoint was also facilitated by the use of two cholesterol preparations labelled in different sites of the molecule (C^{14} 4 cholesterol and C^{14} 26 cholesterol)

The sites of steroid hormone formation were also studied by histochemical localization of hydroxysteroid dehydrogenase activity

The distribution of the two differently labelled cholesterol compounds coincided in most organs which indicated that the radioactivity did not represent steroid hormones formed in the body

Great differences in the kinetics between cholesterol on one hand and pregnenolone and progesterone on the other were registered Accumulation of cholesterol continued for 1—4 days in the adrenal cortex testes and ovaries and then a slow decrease of radioactivity was seen Labelled pregnenolone and progesterone reached their maximum concentrations in adrenal cortex testes and ovaries 2 to 5 minutes after injection The concentration of these two latter hormone precursors then decreased rather rapidly

The difference between cholesterol and the others is probably explained by a more rapid utilization of the latter ones in the synthesis of steroid hormones The chromatographic investigations showed that pregnenolone and progesterone were rapidly metabolized According to their R_f values some of the unidentified radioactive metabolites from endocrinologically active tissues might well have been steroid hormones formed from the labelled precursors Labelled cholesterol was on the other hand to great extent unchanged and/or esterified

The adrenal cortex showed a concentration of labelled material which exceeded that of any ot r tissues in the body after injection of labelled cholesterol The radioac y probably representing cholesterol esters was mainly confined to the zor fasciculata 4 days after i v injection of cholesterol

Labelled pregnenolone was found in the whole adrenal cortex which was also true for Δ^5 3β hydroxysteroid dehydrogenase necessary e g for the conversion of pregnenolone to progesterone Labelled progesterone was also present in all cortical zones which agrees with the finding that 11β and 21 hydroxylase needed for e g transformation of progesterone to deoxycorticosterone and corticosterone are present in all cortical zones (Grant 1962) After injection of labelled progesterone the radioactivity disappeared very rapidly from the adrenal cortex which may indicate a rapid release of the steroid hormones produced

All the precursors studied were taken up in the corpora lutea and Δ^5-3β-hydroxysteroid dehydrogenase activity was also shown to be present in corpora lutea especially in those from pregnant animals The finding of labelled progesterone in corpora lutea may indicate not only a storage but also a precursor role there

In the ovary labelled cholesterol was also taken up in the interstitial tissue theca interna and to some extent in the granulosa cells The concentration of labelled pregnenolone and/or its metabolites was low in other ovarian tissues than corpora lutea However the Δ^5 3β hydroxysteriod dehydrogenase activity with pregnenolone as a substrate was rather strong in the interstitial tissue and some follicles Labelled progesterone and/or its metabolites was observed in some follicles short time after injection

The uptake of the injected precursors and/or labelled hormonal products in uterus was not very pronounced Only pregnenolone showed a specific locali zation in the placenta Labelled cholesterol and progesterone were rather evenly distributed in the placenta

The interstitial tissue in the testes is the predominant source of androgens and C^{14} labelled cholesterol pregnenolone and progesterone were shown to accumulate there but were also seen in a low concentration in the tubuli

There was a very slow uptake of labelled cholesterol in the brain Sixty days after injection an uptake in the white matter of the central nervous system was seen Labelled pregnenolone and progesterone were taken up shortly after inpection and the highest concentration was seen in the grey matter After 10 to 20 minutes there was more radioactivity in the white matter than in the grey matter The uptake of progesterone and pregnenolone is correlated to their previously known actions on the central nervous system

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 302

STUDIES ON THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF CATECHOLAMINES AND THEIR α-METHYLATED ANALOGUES

BY

PER LUNDBORG

GÖTEBORG 1967

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 302

FROM THE DEPARTMENT OF PHARMACOLOGY
UNIVERSITY OF GÖTEBORG SWEDEN

STUDIES ON THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF CATECHOLAMINES AND THEIR α-METHYLATED ANALOGUES

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ELANDERS BOKTRYCKERI AKTIEBOLAG
1967

Apart from certain hitherto unpublished results the present thesis is based on the following papers

1 Storage function and amine levels of the adrenal medullary granules at various intervals after reserpine treatment *Experientia* 1963 *19* 479–480
2 Uptake of metaraminol by the adrenal medullary granules *Acta physiol scand* 1966 *67* 423–429
3 Two different mechanisms for incorporation of ^{3}H metaraminol into the amine storing granules *J Pharm Pharmacol* 1966 *18* 762–764 (Together with B Waldeck)
4 Effect of reserpine and monoamine oxidase inhibition on the uptake and subcellular distribution of ^{3}H noradrenaline *Brit J Pharmacol* 1967 *29* 99–104 (Together with R Stitzel)
5 Uptake of biogenic amines by two different mechanisms present in adrenergic granules *Brit J Pharmacol* 1967 *29* 342–349 (Together with R Stitzel)
6 Effect of reserpine and protriptyline on the subcellular distribution of ^{3}H metaraminol in the mouse heart *Brit J Pharmacol* 1967 *30* 379–384 (Together with R Stitzel)
7 Stereospecificity and intracellular binding of metaraminol *Acta physiol scand* 1967 (in press) (Together with R Stitzel)
8 Studies on the dual action of guanethidine in sympathetic nerves *Acta physiol scand* 1967 (in press) (Together with P Stitzel)

These papers will be referred to below by their arabic numerals (Paper 1 Paper 2 etc)

ABBREVIATIONS USED

A=adrenaline ATP=adenosine triphosphate DA=dopamine 5 HT=5 hydroxytryptamine MA=metaraminol MAO=monoamine oxidase α MeNA=α methyl noradrenaline NA=noradrenaline NM=normetanephrine

$$\frac{P}{P+S} = \frac{\text{particulate fraction}}{\text{particulate fraction}+\text{supernatant fraction}}$$

RNA=ribonucleic acid

INTRODUCTION

Our knowledge about the storage of catecholamines was relatively incomplete until about fifteen years ago Since that time with the development of new techniques many studies on the storage of catecholamines have been conducted both on the adrenal gland and on other tissues and the results have been summarized in numerous reviews (Hagen and Barnett 1960 Bertler *et al* 1960 Green 1962 Stjarne 1964 Schumann 1966 Potter 1966)

Independently Blaschko and Welch (1953) and Hillarp *et al* (1953) isolated catecholamine storage granules from the adrenal medulla using differential centrifugation In 1955 it was observed that these granules contained relatively large quantities of adenosine triphosphate (ATP) (Hillarp *et al* 1955) This finding together with the constant molar ratio of ATP to catecholamines (approximately 1 4) in the granules suggested that ATP might be functioning as an anion in a salt complex with the amines (Blaschko *et al* 1956 Falck *et al* 1956 Carlsson and Hillarp 1956*a* Hillarp 1958*a* 1958*b* 1959)

The precise chemical composition of the granules has been described by Hillarp (1959) as being 68 5% water 6 7% catecholamines 11 5% protein 4 5% adenine nucleotides and 7% lipid Philippu and Schumann (1963) have also found RNA to be present in the medullary granules About 77% of the protein is soluble (Hillarp 1958*a* 1958*b*) Since there are relatively large amounts of the soluble protein it has been postulated that it has some role in the binding of the amines (Hillarp 1959 Helle 1966) The possibility that bivalent ions like Ca^{++} and Mg^{++} as well as RNA may participate in the formation of the storage complex has also been presented (Schumann 1966)

Catecholamine containing storage granules have also been isolated from splenic nerves (Euler and Hillarp 1956 Schumann 1958*a* 1958*b*) brain (Chrusciel 1960) and heart (Campos and Shideman 1962 Potter and Axelrod 1963*a* 1963*b* Schumann *et al* 1964 Glassman *et al* 1965) These granules are much less dense than the chromaffin granules of the adrenal medulla and occur in the microsomal rather than in the mitochondrial fraction Schumann (1958*b*) has demonstrated that granules isolated from sympathetic nerves contain considerable amounts of ATP (40 $\mu g/g$) and that the molar ratio of catecholamines to ATP is about 5 2 1 which is in the same range as the ratio found in granules isolated from the adrenal medulla (approximately 4 1)

Granulated vesicles have been examined in electron micrographs of sympathetically innervated tissues and are presumably characteristic of adrenergic vesicles (Richardson 1962 Hökfelt 1967) Using a combination of autoradiography and electron microscopy Wolfe *et al* (1962) demonstrated that these vesicles were able to take up ^{3}H NA

Carlsson *et al* (1962) as well as Kirshner (1962*a* 1962*b*) found that the *in vitro* uptake of catecholamines into adrenal medullary granules could be increased severalfold by the inclusion of ATP and Mg^{++} in the incubation medium This uptake was temperature dependent and could be blocked by low concentrations (10^{-8} M) of reserpine The relative nonspecificity of the uptake mechanism was shown by the ability of the granules to accumulate several types of biogenic amines such as A NA 5 HT and DA (Carlsson *et al* 1963*a*) As to other amines tyramine and tryptamine are taken up though the mechanism is different (Carlsson *et al* 1963*a* Jonasson *et al* 1964) The possible mechanism involved in this uptake will be further discussed below

Granules isolated from the splenic nerves have also been shown to possess an uptake mechanism which is ATP Mg^{++} dependent blocked by low concentrations of reserpine (Euler and Lishajko 1963*a*) and temperature dependent Heart granules behave somewhat differently in that the amine uptake is only moderately potentiated by ATP and is not inhibited by doses of reserpine which are effective on adrenal and splenic nerve granules Only in an extremely high concentration (10^{-3} M) has reserpine been found to block the uptake of amines into heart granules (Potter and Axelrod 1963*b*) *In vitro* similarities and differences between granules isolated from splenic nerves and adrenal medullary granules with respect to turnover rate resistance to osmotic changes temperature stability etc have been carefully studied and discussed and the possible danger in extrapolating *in vitro* data from adrenal medullary granules to *in vivo* data on nerve granules has been pointed out (Stjarne 1964 1966*a*) That granules from various adrenergic tissues may differ in their *in vitro* properties has been pointed out by Euler (1966*a* 1966*b*)

The functions of the amine granules do not seem to be restricted to uptake and retention of biogenic amines Dopamine β hydroxylase the enzyme responsible for the last step in the synthesis of NA has been found in adrenal granules (Levin *et al* 1960 Kirshner 1962*b* Kirshner *et al* 1963) as well as *in the heart nerve granules (Potter and Axelrod 1963b) Recently dopamine* β hydroxylase has been shown to exist in granules isolated from the splenic nerve (Stjarne 1966*b*)

The uptake of biogenic amines into sympathetic nerves was the subject of an extensive review by Stjarne (1964) Raab and Gigee (1955) first demon

strated that tissues are able to take up and store exogenously administered catecholamines, a finding which has subsequently been confirmed by others *in vitro* on isolated organs (Iversen 1963) on tissue slices (Dengler *et al* 1962 Ross and Renyi 1964 Hamberger and Masuoka 1965) as well as *in vivo* (Muscholl 1960 Whitby *et al* 1961) That these amines are concentrated in adrenergic nerves is suggested by the reduced retention observed after post ganglionic denervation (Hertting *et al* 1961) and immunosympathectomy (Iversen *et al* 1965*a* Klingman 1965)

The localization of the exogenous amine within the adrenergic neuron has also been demonstrated by autoradiographic (Wolfe *et al* 1962) and histo chemical techniques (Hamberger *et al* 1964 Hillarp and Malmfors 1964 Malmfors 1965)

It has been postulated that the uptake mechanism for accumulating amines within sympathetic nerves consists of two major components active transport through the nerve cell membrane and incorporation into the storage granule complex (Carlsson *et al* 1963*a* Hillarp and Malmfors 1964 Malmfors 1965 Carlsson and Waldeck 1965) Both of these mechanisms can be selectively blocked by drugs Protriptyline and desipramine are potent inhibitors of the former mechanism while reserpine is a potent blocker of the latter

The principle aims of the present work were to study the mechanisms *involved in the uptake and accumulation* of biogenic amines both *in vivo* and *in vitro* and to study the subcellular sites of action of drugs acting on the sympathetic nervous system

The principal methods used in the present study have been studies *in vitro* on isolated adrenal granules and studies *in vivo* on uptake and distribution of tritiated amines into the mouse heart

The adrenal granules were prepared in a manner similar to that described by Hillarp (1958*a*) The rabbit adrenal glands were removed immediately after death and the granules were prepared in the cold Bovine adrenals were cut out within 30 min after death placed in the cold and the granules prepared as soon as possible The medullas were dissected out cut into small pieces and homogenized in 0 3 M sucrose in a glass homogenizer with a loose fitting plastic pestle Unbroken cells and nuclei were removed by centrifugation at 800×g for 5 min The supernatant was decanted and centrifuged at 26 000×g for 20 min The supernatant was decanted and the looser layer above the more tightly packed bottom sediment was removed by swirling with sucrose Finally the granules were suspended in 0 3 M sucrose and stored at 0 C They were used on the same and during the next two days

The uptake capacity of the granules seemed to vary in different experiments One reason for this could be due to variations in time between sacrifice of the cows and removal of the adrenals Another cause of variable uptake capacity may be that the granules used on the second day post sacrifice may have undergone some changes For this reason controls were run in each experiment Also in all rabbit experiments control animals were run together with treated animals

The standard conditions for incubation experiments were as follows In rabbit experiments granules prepared from the two adrenals of one animal were added to a single incubation vial In experiments with bovine granules an aliquot (50μl) of the granule suspension—in most experiments corresponding to 115–125 μg catecholamines 20–25 μg of which are free amines released from the granules at their suspension—was transferred to 1 0 ml of an incubation mixture (at 0 C) prepared as recommended by Carlsson *et al* (1963*a*) The buffer used was 0 31 M glycylglycine (ph 7 3) and in most experiments ATP (0 0025 M) and MgCl (0 0025 M) were also included Varying concentrations of labelled amines were added If not otherwise stated corrections for the contribution of amines released from granules were not made Incubations

were performed without shaking at +31°C and 0°C (controls) for 30 min Practically all tests were made in duplicate A detailed description of the rest of the procedure is given in Papers 1 and 2

All *in vivo* experiments in the present study were conducted on groups of mice each group consisting of six animals After sacrifice of the animals by decapitation the hearts were removed and immediately homogenized in approximately 10 volumes of 0 25 M sucrose containing 0 005 M phosphate buffer (pH 7 4) and 0 001 M MgCl The latter two substances were added to the medium in accordance with the suggestions of Potter and Axelrod (1963*b*) The homogenization was performed in an ice bath using a glass homogenizer and a loose fitting plastic pestle The speed in air of the pestle was 1000 r p m but during homogenization there was a small reduction of speed due to resistance The number of strokes up and down during homogenization was always 60 and to avoid unnecessary variations the same technical assistant performed all homogenizations

A coarse fraction was removed by centrifugation of the homogenate in the cold at 2000 × g for 10 min The supernatant obtained was then centrifuged at 100 000 × g for 60 min in a Spinco Model L ultracentrifuge providing two more fractions particulate (sediment) and high speed supernatant Further details of the analytical procedure are given in Papers 4 and 5

In control experiments a quantity of ^{3}H NA ^{3}H α MeNA or ^{3}H MA was added to samples of cardiac tissue immediately before homogenization and a subcellular distribution was then performed Only 3 per cent of the total activity found in the particulate and supernatant fractions was recovered in the former fraction Thus in the present experiments any uptake of the amines by the particulate fraction above 3 per cent must have occurred *in vivo*

To avoid the effects of hypothermia all animals were kept at 30 C

In tests where internal standards were added to the various fractions the recovery after column procedure was 88±2% No corrections for recovery were made

The homogenization of the cardiac tissue was not 100 per cent complete Histochemical pictures of the coarse fraction (Jonsson personal communication) show that there are several intact axon parts with unbroken varicosities exhibiting fluorescence typical of catecholamines However homogenization with more than 60 strokes does not result in any further increase in the amount of granules recovered (Table I)

Alterations in the pH of the buffer in the homogenizing medium between pH 7 and 8 did not change the release of labelled amines from the particulate fraction (Potter and Axelrod 1963*b* Lundborg unpublished) However it was felt desirable to keep the pH at a constant level

Table I Distribution of ^{3}H α MeNA in subcellular fractions of the mouse heart after various numbers of strokes during the homogenization procedure ^{3}H α meNA (100 μg/kg i v) was given 15 min before sacrifice of the animals

Number of strokes	Distribution in %			$\frac{P}{P+S} \times 100$
	Coarse fract	Partic fract	Supern fract	
20	47 4	17 9	34 7	34 0
40	33 7	26 8	39 5	40 4
60	30 4	30 9	38 6	44 4
80	29 9	29 6	40 5	42 1
100	32 8	29 9	37 3	44 4
120	32 5	30 3	37 1	44 9

Potter (1966) has described various ways to obtain a granule fraction as pure as possible However the aim of the present work was not to get a 100 per cent pure nerve granule fraction but rather a particulate fraction which included as many of the granules present in the neuron as possible Even if the particulate fraction prepared in the present work was contaminated with mitochondria Potter and Axelrod (1963*a*) have shown that the mitochondrial fraction does not contain any of the labelled amine used in their experiments

Histochemical pictures of the particulate fraction show only a few fluorescing spots which presumably represents pinched off nerve endings but apart from this the fraction appeared quite homogeneous

A Effect of reserpine on uptake and storage of catecholamines

(Papers 3 and 4)

Reserpine is known to cause depletion of catecholamines from adrenal glands (Carlsson and Hillarp 1956*b*) the cat hypothalamus (Holzbauer and Vogt 1956) and sympathetically innervated organs (Bertler *et al* 1956 Muscholl and Vogt 1958) It was originally suggested (Carlsson *et al* 1957) that the depletion of catecholamines produced by reserpine was due to amine release immediately followed by deamination the latter phenomenon probably being an intracellular event It was further suggested that reserpine might also produce an inhibition of an active storage mechanism (Carlsson *et al* 1957 Muscholl 1960) A direct blocking effect of reserpine on the adrenal medullary granules has been shown *in vivo* (Bertler *et al* 1961) and *in vitro* (Carlsson *et al* 1962 1963*a*, Kirshner 1962*a* 1962*b*) as well as *in vitro* on splenic nerve granules (Euler and Lishajko 1963*a*) (Review see Carlsson 1965)

However it has also been found that reserpine in low concentrations inhibits the spontaneous release of NA from isolated splenic nerve granules (Euler and Lishajko 1961 1963*b* Stjärne 1966*a*) Whether a similar effect occurs *in vivo* is not yet known

Campos and Shideman (1962) observed that pretreatment with reserpine impaired uptake of NA into the dog heart and especially into the particulate fraction but apart from this work few *in vivo* studies are available demonstrating an action of reserpine on subcellular amine distribution *in vivo*

After homogenization much of the NA recovered from the supernatant fraction under normal conditions is probably an artefact representing release of the amine from the particulate fraction during homogenization It is now generally accepted that most of the intraneuronal NA is particle bound (Euler 1966*a* Potter 1966) This is supported by the observation that the supernatant ^{3}H NA content of the normal heart does not show much decrease with time suggesting that *in vivo* it was bound in some manner and therefore protected from intraneuronal MAO (Paper 4)

In vivo uptake and subcellular distribution of ^{3}H NA ^{3}H α MeNA *and* ^{3}H MA *after reserpine treatment*

In Paper 4 the subcellular distribution of injected ^{3}H NA in the heart of reserpine treated mice was studied both with and without MAO inhibition

Untreated animals and animals treated with MAO inhibitor only were used as controls. Nialamide in a dose of 10 mg/kg was injected 2 hr before ^{3}H NA administration since this has been found to be the optimal condition for measuring the accumulation of injected ^{3}H NA in reserpine treated mice (Carlsson and Waldeck 1967).

In untreated animals and in animals treated with nialamide alone ^{3}H NA was taken up in the heart to almost the same extent and the ^{3}H NA was about equally distributed between the particulate and the supernatant fractions at all time intervals studied. Treatment with reserpine alone resulted in a greatly diminished accumulation of ^{3}H NA both in the particulate and in the supernatant fractions. Reserpine treated animals which had also been given nialamide still showed a low concentration of ^{3}H NA in the particulate fraction but there was an appreciable accumulation in the supernatant fraction. This latter accumulation was particularly marked at the 15 min [illegible]

The low levels of ^{3}H NA found after reserpine treatment could [illegible] a rapid destruction by MAO. If reserpine acts by inhibiting the [illegible] the granules the injected ^{3}H NA would tend to accumulate [illegible] and thus be easily broken down by the enzyme. In support [illegible] was found that following administration of reserpine and a [illegible] the supernatant fraction contained appreciable amounts of ^{3}H [illegible]

Even when the ^{3}H NA is protected from enzymatic destr[illegible] there is a rapid disappearance of the amine in animals pretreated [illegible] All of the extragranular ^{3}H NA which had accumulated was [illegible] min. There are various possible explanations for this. One [illegible] the inhibition of MAO is not complete even if the condition[illegible] Also nialamide may have some releasing action of its own [illegible] by Carlsson and Waldeck (1966*a*) and further investig[illegible] Lindqvist and Waldeck (in preparation) in the presence [illegible] endogenous amines may accumulate which displace the ^{3}H [illegible] granular sites.

In Paper 4 the amount of labelled NM recovered from [illegible] studied. The amount of ^{3}H NM increased after reserpine [illegible] due to a decreased capacity of the adrenergic nerves to [illegible] posing it to increased enzymatic destruction. It was [illegible] not bound to the particulate fraction and that it rap[illegible] the tissues.

Another way to demonstrate an amine accumulation [illegible] in reserpine treated animals is to use an amine res[illegible] a MeNA or MA. ^{3}H MA data are presented in Paper 3 and [illegible] is shown in Fig. 1 (cf. also Paper 5).

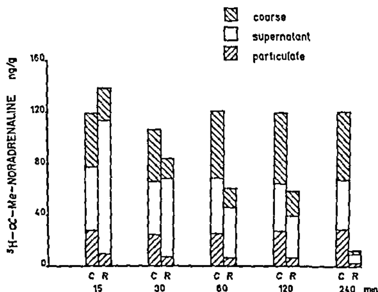


Fig 1 Uptake of ^{3}H α MeNA by subcellular fractions of the mouse heart Animals were given ^{3}H α MeNA (100 μg/kg i v) alone (left bar) or preceded by reserpine (10 mg/kg i p 6 hr before right bar) Each bar represents the mean of 6 experimental groups consisting of 6 animals per group (From Carlsson Lundborg Stitzel and Waldeck 1967)

Reserpine treated (10 mg/kg) and control mice were given ^{3}H α MeNA (100 μg/kg) or ^{3}H MA (40 μg/kg) i v The animals were sacrificed at various time intervals after amine administration and subcellular distribution was performed on cardiac tissue In control animals the total amount of ^{3}H α MeNA in the heart and the amount in each subcellular fraction remained relatively constant for at least 4 hr The uptake and distribution of ^{3}H α MeNA into subcellular fractions was quite rapid being accomplished within 15 min There was an indication however of a gradual redistribution of ^{3}H α MeNA in favour of the particulate fraction during the 4 hr period studied This was shown by an increase in the P/(P+S) ratio from 36 to 43% For ^{3}H MA this redistribution was still more apparent changing from 14 5% to 32 7% during the 8 hr period studied The total amount of ^{3}H MA in the heart was almost constant during this time

Pretreatment with reserpine did not affect the initial uptake but markedly impaired the ability of the heart to retain both ^{3}H α MeNA and ^{3}H MA Four hr after the tritiated amine was given to reserpine treated animals the total retention of ^{3}H α MeNA was only about 10% of that seen in the corresponding control A subcellular distribution revealed that reserpine administration resulted in a reduced uptake into the particulate fraction

with a concomitant increase in the supernatant fraction The rapid disappearance of ^{3}H α MeNA from reserpine treated hearts appeared to be due to a continual loss from the supernatant fraction while the content of the particulate fraction remained relatively stable although reduced

The retention of ^{3}H MA also was impaired after reserpine treatment The concentration fell from 26 0 ng/g at the 30 min interval to 7 6 ng/g after 4 hr Reserpine only slightly altered the distribution of ^{3}H MA at the 30 min interval but the further incorporation of ^{3}H MA into the particulate fraction was blocked It is interesting to note that even after a large dose of reserpine significant amounts of ^{3}H α MeNA as well as ^{3}H MA could still be taken up and retained in the particulate fraction This reserpine resistant uptake will be discussed later

The results of ^{3}H α MeNA and ^{3}H MA uptake studies are similar to earlier observations made with ^{3}H MA (Carlsson and Waldeck 1965) Unlike NA the total accumulation of the α methyl analogues in the tissues of reserpine treated mice was about the same as in control animals Probably all three amines are taken up by a mechanism located at the level of the cell membrane of the adrenergic nerves but NA is rapidly destroyed by intraneuronal MAO The α methyl analogues are resistant to MAO and can thus more easily accumulate inside the nerve cell However the ability of the nerves to retain these analogues is very much reduced by reserpine The reason for this is evident from the subcellular distribution studies Reserpine inhibits the amine incorporation into the particulate fraction The amines thus accumulate in the extragranular cytoplasm where they cannot be efficiently retained possibly due to leakage through the nerve cell membrane

It can be concluded from these experiments that reserpine acts *in vivo* by blocking the incorporation of amines into the granules These findings are in agreement with earlier *in vitro* data on adrenal medullary and splenic nerve granules

B Uptake of catecholamines at various intervals after reserpine treatment

(Paper I)

Reserpine not only depletes the catecholamine stores but also reduces the response of various organs to sympathetic nerve stimulation (Carlsson *et al* 1957 Muscholl and Vogt 1958) It is also well known that after a single injection of a large dose of reserpine to animals the pharmacological effects (sedation miosis ptosis) disappear within the first two days whereas the levels of tissue catecholamines still remain low

with ice and homogenized Subcellular distribution extractions and further procedures were performed as described in Methods section

When ^{3}H NA was given to animals pretreated with reserpine, there was an almost complete blockade of uptake of the tritiated amine into the particulate fraction lasting up to 12 hr (Fig 2) During the next 12 hr there was a small but gradual increase in the uptake of ^{3}H NA into the granules while between 24 and 48 hr after reserpine administration a marked increase in amine uptake was observed Thereafter a continual recovery of the granular uptake function occurred until at 48 and 96 hr the uptake of the granules was about 35 and 80% of normal respectively

The uptake of ^{3}H α MeNA by the adrenergic granules was reduced to about 30% of control within 30 min after a single reserpine injection (Fig 2) This degree of inhibition remained relatively constant for up to 12 hr and then gradually diminished during the next 12 hr period Between 24 and 48 hr after reserpine administration there was a marked increase in the ability of the granules to take up ^{3}H α MeNA until at 48 hr the uptake was approximately 70% of normal When the amount of labelled amine in the particulate fraction was expressed as a percentage of that found in the particulate plus supernatant fractions (P/(P+S)×100) ^{3}H NA was found to be present in a slightly greater concentration (45%) than was ^{3}H α MeNA (42%) Mice pretreated with reserpine however showed a greatly reduced distribution ratio This effect was particularly marked during the first 12 hr following the administration of reserpine and then gradually subsided (In the case of ^{3}H NA the distribution ratios recorded during the first 12 hr after reserpine were not very reliable owing to the small amounts present During this period more emphasis should thus be placed on the ^{3}H α MeNA data) The distribution of ^{3}H NA had returned essentially to normal 48 hr after the injection of reserpine while that for ^{3}H α MeNA did not return to normal until 96 hr (Fig 3) This difference between the two amines is based on reliable data and calls for a comment

During the first few hours after reserpine administration almost all ^{3}H NA taken up by the nerve cell is probably rapidly destroyed by the intracellular MAO The first signs of recovery in the particulate fraction noticed after 18–24 hr would indicate the first recovery of a small granular fraction This fraction is apparently not large enough to take care of all ^{3}H NA taken up by the nerve cell membrane pump but evidently is enough to prevent a certain amount of the amine from being destroyed This destruction of extragranular ^{3}H NA by MAO probably explains why an approximately normal distribution ratio is obtained already 18 24 hr after reserpine

^{3}H α MeNA is resistant to MAO and thus there is an accumulation of

the amine in the sympathetic nerve even if granular uptake mechanisms are impaired Thus the P/(P+S) ratio is not back to control values until the uptake function is fully recovered

It can be concluded that a certain amount of granular function is recovered already 18–24 hr after reserpine treatment However the time needed for complete recovery in the granular uptake function seems to be at least 96 hr

The recovery of granular uptake after reserpine treatment may be explained by the formation of new storage granules within the sympathetic nerve and their subsequent transport down the axons to the nerve terminals (Dahlstrom 1965 Dahlstrom and Haggendal 1966) The studies of Dahlstrom (1966 1967) have shown that fresh uninhibited granules have begun to reach the nerve terminals within 24 hr after reserpine administration However the possibility of a reversal of the reserpine induced inhibition cannot be ruled out Recovery of an important granular pool 24–48 hr after a large dose of reserpine could also account for the return of granular uptake It was not possible in the present study to distinguish between these two possibilities

It is for the moment difficult to state anything about the possible interrelationship between the relatively small granular pool which may be essential for function and the small labile ATP free granular pool observed by Hillarp (1960)

C Uptake and release of metaraminol

In vitro studies (Papers 2 5 and 6)

The uptake of catecholamines and 5 HT by the ATP Mg^{++} dependent uptake mechanism present in adrenal medullary granules was carefully investigated by Carlsson *et al* (1963*a*) Since MA has proved to be a very useful tool in pharmacological experiments it was also felt desirable to study the ability of the granules to incorporate this α methylated analogue It was soon found that MA behaved differently from the catecholamines in a number of ways

The uptake of MA into the granules was not saturated until a concentration above $2\times10^{-}$ M was used The corresponding figure for A uptake was found by Jonason *et al* (1964) to be about $4–5\times10^{-4}$ In some experiments with MA ATP and Mg^{++} were excluded from the incubation medium This resulted in no or at most a slight decrease in the uptake of MA Reserpine is a potent blocker of the ATP Mg^{++} dependent uptake of catecholamines However even in relatively high concentrations reserpine was unable to block the uptake of MA to any large degree In some experiments reserpine had

no blocking effect at all while in others (Papers 5 and 6) it had only a slight blocking effect

From these data it was suggested that MA *in vitro* is taken up by the adrenal medullary granules by a mechanism different from that utilized by the catecholamines

It was shown that the uptake of MA was accompanied by the release of equivalent amounts of catecholamines from the granules The incorporation of tyramine into the adrenal granules by a displacement of the endogenous catecholamines from their storage sites has been pointed out earlier (Schumann 1960 Schumann and Philippu 1961 1962) At least two different mechanisms for the *in vitro* uptake of amines into the adrenal granules seem to exist The first an ATP Mg^{++} dependent uptake is blocked by reserpine and the second is a mechanism dependent on displacement of endogenous amines The former mechanism is the one preferentially utilized by catecholamines the latter by MA and tyramine

When A was incubated together with increasing concentrations of MA a gradually increasing inhibition of the A uptake was observed To study the nature of this inhibition various amounts of A were incubated together with 2 different concentrations of MA The values obtained were plotted according to the method of Lineweaver and Burk (1934) It was found that MA bitartrate in a concentration of 200 μg/ml acts mainly as a competitive inhibitor of the A uptake The K_m value for A was estimated at $4\ 3\times10^{-4}$ M which is in agreement with earlier findings (Jonason *et al* 1964) The K_i value for MA was calculated at $5\ 2\times10^{-4}$ M Thus MA and A have almost the same affinity for the ATP Mg^{++} dependent uptake mechanism but only A seems able to utilize it to any larger extent *in vitro*

The possible importance of stereospecificity in the uptake of MA was also studied A constant concentration of ^{3}H *dl* MA was incubated with varying concentrations of unlabelled *l* or *d* MA Dilution with the *l* form reduced the uptake of ^{3}H MA more than dilution with the *d* form A possible explanation is that the uptake of MA though not absolutely stereospecific has a preference for the *l* form

In vivo studies (Papers 3 6 and 7)

The *in vitro* findings that the MA uptake into adrenal granules was virtually unaffected by reserpine were slightly confusing in the light of *in vivo* studies by Shore *et al* (1964) and Carlsson and Waldeck (1965) which showed that the ability of the rat and mouse heart respectively to retain MA was considerably inhibited by reserpine It was felt desirable to investigate further this apparent discrepancy between *in vitro* and *in vivo* evidence

Exogenously administered MA can accumulate in sympathetically innervated tissues with the subsequent displacement of endogenous NA (Andén 1964 Shore *et al* 1964) The MA taken up accumulates within adrenergic nerves (Jonsson and Ritzén 1966) and can be released by nerve stimulation (Crout *et al* 1964 Almgren *et al* in preparation) However it is not taken up by hearts of immunosympathectomized animals (Shore *et al* 1964) or after treatment with a drug which selectively blocks the membrane pump of adrenergic nerves such as desipramine (Carlsson and Waldeck 1965)

At least part of the MA taken up is bound to subcellular particles in the heart (Giachetti and Shore 1965)

Effect of denervation on the in vivo uptake of ^{3}H MA

The following experiments were performed to further demonstrate that most of the ^{3}H MA present in the particulate fraction is localized in the post ganglionic sympathetic neurons

Male Sprague Dawley rats were used The submaxillary glands were post ganglionically sympathectomized by excision of the superior cervical ganglion In all cases the operation was unilateral the intact side serving as a control The animals were injected with ^{3}H MA (40 μg/kg) i v 5–10 days after the

Table II Uptake and retention of ^{3}H MA by subcellular fractions of intact and denervated rat salivary glands ^{3}H MA (40 μg/kg i v) was given 30 min or 24 hr prior to sacrifice of the animals All data in each row are from the same animal ^{3}H Ma values are expressed as ng/g tissue

Coarse fraction			Particulate fraction			Supernatant fraction		
Intact gland	Denerv gland	$\frac{Den}{Int}$ × 100	Intact gland	Denerv gland	$\frac{Den}{Int}$ × 100	Intact gland	Denerv gland	$\frac{Den}{Int}$ × 1[illegible]
0 min								
40 [illegible]	8 0	19 7	8 0	0 6	[illegible]4	46 [illegible]	8 5	18 3
30 9	8 6	7 7	7 [illegible]	0 9	11 4	46 2	10 7	3 2
3 4	14 0	19 1	16 7	1 6	9 7	[illegible]7 0	19 7	25 0
		2 [illegible] ± [illegible]7			9 5±1 16			[illegible] 4±2
24 hr								
13 2	0 4	2 9	3 3	0 1	1 8	10 1	0 4	4 3
11 8	0 2	1 5	1 [illegible]	0 0	0 0	8 3	0 2	2 [illegible]
10 3	0 6	[illegible]3	1 6	0 1	3 0	6 6	0 4	6 [illegible]
		3 ±1 11			1 6±0 87			4 6±1 [illegible]

operations and were killed either 30 min or 24 hr later The submaxillary glands were removed and treated similar to the method described in the Results section for cardiac tissue However fewer strokes (30) were used for homogenization The ^{3}H MA content of the coarse and supernatant fractions was about 20% and the particulate fraction about 10% of the corresponding amounts in the intact gland (Table II) There was almost no ^{3}H MA left in the denervated glands 24 hr after ^{3}H MA administration

Effect of reserpine and protriptyline on the uptake and release of ^{3}H MA

If ^{3}H MA was injected into mice the heart content remained at about the same concentration for at least 8 hr During this time the amount recovered from the particulate fraction increased gradually the P/(P+S) changing from 14 5% to about 33% If the animals were pretreated with reserpine the up take of ^{3}H MA was almost as efficient as in untreated animals during the first 30 min However the amine was less efficiently retained The P/(P+S) ratio in reserpine treated mice did not increase as in untreated animals but was kept almost constant during the time period studied Apparently an intragranular mechanism sensitive to reserpine is necessary for efficient retention of ^{3}H MA in sympathetic nerves

The nature of the MA uptake mechanisms could be further elucidated through studies on the releasing effect of reserpine and protriptyline The latter drugs were administered i v 15 min or 24 hr after an ^{3}H MA injection and the animals were killed 45 min later Some groups given the releasing drugs 15 min after ^{3}H MA injection were killed after another 15 min

Reserpine had no significant ability to reduce the amount of ^{3}H MA which was retained in the hearts during the first 30 min after the injection of the amine However reserpine was able to deplete ^{3}H MA which was given 1 hr or 24 hr previously Reserpine was particularly effective when given 24 hr after the intravenous administration of ^{3}H MA The primary site of depletion was the particulate fraction the concentration of ^{3}H MA in the supernatant fraction remaining essentially unchanged

From the data given it can be suggested that the ^{3}H MA *is first taken up in the granules by a mechanism which is resistent to reserpine and there after it is either taken up in or transferred to a reserpine sensitive mechanism from which it can easily be released by reserpine*

Protriptyline induced a loss of the labelled amine from the supernatant fraction at the shorter intervals after ^{3}H MA administration while there was *no significant decline in the particulate fraction* When it was given 24 hr after the labelled amine protriptyline had a much smaller effect on the

content of ^{3}H MA remaining in the supernatant fraction The particulate fraction was not affected

Under normal conditions blockade of the membrane pump by protriptyline does not cause a depletion of NA (Carlsson and Waldeck 1966b Malmfors 1965) However Carlsson (1966a) has pointed out that if there is a sufficient accumulation of the transmitter or one of its analogues in the axoplasm and then the membrane pump is blocked a rapid spontaneous release of axoplasmic amine occurs which is not compensated for by a functioning membrane pump Apparently ^{3}H MA shortly after its administration is accumulated in sites from which it can be easily released by protriptyline but is gradually transferred to other intracellular pools where it is less available It must be pointed out however that there is a difference in behaviour between MA and other amines like NA and α MeNA It is much easier to release MA than the other amines The concentration of free MA in the cytoplasm is probably higher and MA is more lipid soluble than NA and α MeNA It therefore *diffuses more easily across the cell membrane (Carlsson 1966b)*

That protriptyline acts specifically on the cell membrane and not on the storage granules is further supported by the *in vitro* data which demonstrate that protriptyline even in high concentrations does not inhibit the uptake of ^{3}H MA by isolated adrenal granules

Effect of d and l MA *on the release of* ^{3}H MA

The stereospecificity of the uptake of metaraminol was studied by administering labelled *dl* MA and observing the subcellular distribution of this amine after later administration of unlabelled *d* and *l* isomers The two isomers of MA were given to mice either 15 min or 24 hr after ^{3}H *dl* MA administration and the animals were killed after 15 min

Fifteen min after the injection of the labelled racemic mixture both of the unlabelled isomers displaced ^{3}H *dl* MA from subcellular fractions but *l* MA was the more effective displacing agent Twenty four hr after the administration of ^{3}H *dl* MA however the unlabelled *d* isomer was virtually incapable *of displacing the labelled amine from any subcellular site* while the *l* form was still effective

Shore *et al* (1964) have shown that injected *d* MA is essentially eliminated 3 hr after its intravenous administration while the *l* isomer is retained for many days In the light of these studies it is not probable that in the present experiments any ^{3}H *d* MA remained for exchange with the unlabelled isomer at the 24 hr interval The ^{3}H *l* MA remaining was apparently not in a site readily accessible to the *d* form

It is interesting that *l* MA was more effective in displacing ^{3}H MA from the particulate fraction at the shorter time interval while reserpine was a more effective displacing agent at the longer interval These data are in agreement with the suggestions given above that there is a transfer of MA from one granular pool to another That is there seems to be a movement of ^{3}H MA from one granular pool from which it is easily released by *l* MA to another storage site where reserpine is a more effective displacing agent

From the results referred to here it is also difficult to find support for the view that reserpine *in vivo* acts by inhibiting the release of amines from the granules If this were true it would be difficult to explain the reserpine induced release of ^{3}H MA from the particulate fraction It seems difficult at present to find an explanation for the different behaviour of adrenergic nerve granules in *in vitro* and in *in vivo* experiments On the whole one has to be cautious in making conclusions about *in vivo* conditions from data obtained *in vitro*

The data given above indicate that the two isomers probably differ in their affinity for binding sites and/or uptake mechanisms It seems as if both *d* and *l* MA can be taken up by subcellular fractions of the heart but that the *d* isomer is not firmly bound to the particulate fraction and is rapidly lost from sympathetically innervated tissues This is in agreement with the *in vitro* data that the uptake of MA by adrenal granules favoured the *l* form

D Two different uptake mechanisms present in adrenergic granules

(Paper 5)

The existence of a reserpine resistant uptake mechanism in addition to the earlier known reserpine sensitive uptake mechanism present in adrenergic granules was demonstrated by the *in vitro* data on adrenal medullary granules as well as by the *in vivo* data on heart nerve granules It was found desirable to summarize all these data and study the *in vivo* and *in vitro* interaction of adrenergic granules with three structurally related compounds NA α MeNA and MA The *in vitro* studies showed that the incorporation of NA into bovine granules could be almost completely inhibited by reserpine and was strongly potentiated by ATP and Mg^{++} MA incorporation was almost unaffected even in the presence of concentrations of reserpine which completely inhibited NA uptake and was virtually independent of ATP and Mg^{++} α MeNA uptake was greatly inhibited if ATP and Mg^{++} were excluded from the incubation medium and the addition of reserpine only partially inhibited the incorporation of α MeNA The uptake of all three compounds was temperature dependent

In the *in vivo* experiments it was found that the three amines studied

differed in the amount incorporated into the particulate fraction of the mouse heart NA was incorporated to the largest extent and MA the least

After reserpine pretreatment there was a greatly diminished uptake of ^{3}H NA and ^{3}H α MeNA into the particulate fraction accompanied by an appreciable accumulation in the supernatant fraction In the case of ^{3}H MA reserpine only slightly altered the distribution at the 30 min interval The accumulation of ^{3}H NA could be clearly demonstrated only in animals which had been pretreated with malamide

Reserpine did not completely prevent accumulation of any of the labelled amines into the particulate fraction This uptake into the particulate fraction in reserpine treated animals illustrates that a reserpine resistant storage mechanism can operate *in vivo*

It has previously been argued that certain sympathomimetic amines are retained less efficiently by the granular fraction because they lack one or more of the three hydroxyl groups of NA (Musacchio *et al* 1965) The present observations with MA would appear to support this conclusion However this cannot be the entire explanation since we have found that α MeNA which is identical with NA except that it has an additional methyl group is also less efficiently retained The present results show the existence of two different uptake mechanisms in the adrenergic granules These two mechanisms probably differ in their structural requirements

It can be concluded that the principal incorporation of NA and MA occurs by different mechanisms and that α MeNA with structural properties which are related to both NA and MA seems to be able to utilize both uptake mechanisms A reserpine resistant uptake of α MeNA into an intraneuronal structure which might be the amine storage granules has also been observed histochemically (Hamberger and Malmfors 1967) There are several reports on an uptake not dependent on the presence of ATP and Mg^{++} — for example the incorporation of tyramine tryptamine and other amines into nerve or adrenal medullary granules These various reports cannot be compared directly and further investigations will be necessary to elucidate whether it is the same mechanism acting in all cases

E Effects of guanethidine on the dual uptake of amines

(Paper 8)

With the techniques developed during the previous studies it was possible to test the subcellular sites at which various drugs interfere with catecholamine uptake and release The effects of benzquinamide tetrabenazine prenylamine and epsilon amino caproic acid have been studied and will be published else

where An example of how these techniques can be used is seen in the analysis of the subcellular sites of action of guanethidine as presented in the present summary

In vitro studies demonstrated that guanethidine in high concentrations (5×10^{-3} M) caused a relatively pronounced blockade of the ^{14}C A uptake into bovine adrenal medullary granules

In vivo studies demonstrated that guanethidine caused a pronounced blockade of the uptake of ^{3}H α MeNA into the heart during the first 4 hr after guanethidine administration At the 12 hr interval the uptake had partially recovered and after 24 hr the total uptake of ^{3}H α MeNA was almost the same as in control animals This impairment in uptake was probably due to a blockade of the membrane pump rather than a blockade at the granular level since a drug such as reserpine which specifically inhibits the uptake into adrenergic granules under the conditions used here does not block the total uptake of ^{3}H α MeNA into the sympathetic nerve cell but markedly alters the subcellular distribution of the labelled amine (Fig 1) Also after guanethidine administration there was a moderate change in the subcellular distribution of ^{3}H α MeNA consisting of a partial redistribution of the labelled amine from the particulate to the supernatant fraction The alteration in the distribution of ^{3}H α MeNA was most pronounced at the 12 hr interval but was still evident 24 hr after the guanethidine administration

Apparently guanethidine *in vivo* can block the cell membrane pump as well as the granular uptake mechanism but the granular blockade is of longer duration Lindmar and Muscholl (1964) and Shore and Giachetti (1966) have previously presented *in vitro* data which are in agreement with these results

Guanethidine was able to release previously administered ^{3}H MA from both the particulate and supernatant fractions 15 min as well as 24 hr after administration of the labelled compound Reserpine was without effect at the earlier interval but reduced the ^{3}H MA content of the particulate fraction at the longer interval Protriptyline had no effect on the particulate fraction at any time interval However it reduced the amount of ^{3}H MA in the supernatant fractions A combination of reserpine plus protriptyline induced an almost complete release of ^{3}H MA both 15 min and 24 hr after administration of the labelled amine

The effect induced by guanethidine upon the particulate fraction is probably due to a reserpine like action of the drug The total depletion seen after guanethidine appears to correspond more closely to a combined regimen of reserpine and protriptyline *i e* simultaneous blockade of both membrane pump and granular concentrating mechanisms

Guanethidine had no inhibiting effect *in vitro* until very high concentrations were administered However it is possible that in order for guanethidine to exert its granular blocking action *in vivo* quite high intracellular concentrations must be achieved An active transport of guanethidine into adrenergic neurons could help achieve this condition That guanethidine is actively transported into the cell is supported by the finding that blockade of the membrane pump greatly reduces the accumulation of ^3H guanethidine (Brodie *et al* 1965)

The additional blocking action of guanethidine on the membrane pump could explain how the NA release induced by guanethidine but not reserpine may cause sympathomimetic effects The re uptake of the released amines cannot be accomplished by the membrane pump after guanethidine treatment and thus the NA is released in higher concentrations onto receptor sites

CONCLUSIONS

Most of the previous studies on adrenergic nerve granules have been performed *in vitro*. Some of the data obtained have been hard to reconcile with *in vivo* observations on total levels of endogeneous amines. It was important therefore in combination with *in vitro* studies also to perform experiments where all processes studied took place *in vivo*.

Investigations of this kind are difficult to perform with NA since the extragranular fraction of NA is rapidly broken down by MAO. Even if the particulate/supernatant ratio is about 1:1 after homogenization the extragranular fraction of NA is always small. Furthermore, the use of MAO inhibitor+^{3}H-NA may lead to certain unwanted effects as release of the labelled amine tested by accumulated endogenous amines or other effects of the MAO inhibitor *per se*. For this reason, α-methylated amines resistant to MAO were found extremely valuable. The use of these amines has made several new observations possible.

Both *in vitro* (adrenal medullary granules) and *in vivo* (mouse heart) methods were employed in the present experiments.

Both *in vivo* and *in vitro* reserpine is a potent inhibitor of the uptake of ^{3}H-NA and ^{3}H-α-MeNA into the amine storage granules. After reserpine pretreatment there is a pronounced blockade of the uptake of administered ^{3}H-NA and ^{3}H-α-MeNA into the granules with a subsequent extragranular accumulation of the amines. The accumulation of ^{3}H-NA could be demonstrated only after pretreatment with a MAO inhibitor.

In vivo administration of reserpine to rabbits blocks the *in vitro* uptake of catecholamines into the adrenal granules for 12–24 hr. After 48 hr the uptake function is restored while the amine levels of the granules are still very low. It is suggested that the granular uptake function rather than the amine levels are important for adrenergic nerve function.

The *in vivo* incorporation of ^{3}H-NA and ^{3}H-α-MeNA into the heart nerve granules is considerably blocked for 12 hr after a large dose of reserpine. It starts to recover after 12–24 hr but is not completely recovered until after 96 hr.

The uptake of ^{3}H-MA into granules is in part different from the uptake of ^{3}H-NA and ^{3}H-α-MeNA. *In vitro* the uptake of ^{3}H-MA is only slightly

affected by reserpine and is not dependent on the presence of the cofactors ATP and Mg^{++} It is suggested that ^3H MA is taken up into the granules by displacement of endogenous amines *In vivo* ^3H MA is taken up in the granules by a mechanism resistant to reserpine and is then gradually transferred to another storage site This transfer of ^3H MA from one pool to another can be blocked by reserpine and is essential for an effective retention of ^3H MA in the sympathetic nerves Further evidence for the theory that MA is transferred from one pool to another are 1 Reserpine effectively releases ^3H MA from the granular fraction of the heart at long intervals after administration of the labelled amine and is less effective at short intervals 2 In contrast *l* MA is a more effective releasing agent at shorter intervals 3 Protriptyline is most effective in releasing ^3H MA from the heart at short intervals after administration of the labelled amine the site of action is the supernatant fraction

Both *d* and *l* MA can be taken up by subcellular fractions of the heart However the *d* isomer is not firmly bound to the particulate fraction

The possible existence of two different mechanisms for incorporation of biogenic amines into adrenergic granules one reserpine sensitive and one reserpine resistant is pointed out

Evidence is presented indicating that guanethidine inhibits both the transport of amines through the nerve cell membrane and an uptake mechanism present in the amine storing granules The depletion of ^3H MA seen after guanethidine administration may result from a reserpine like action on the amine storage granules and a protriptyline like action on the membrane pump

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STUDIES ON

BLOOD FLOW DISTRIBUTION AND COUNTERCURRENT EXCHANGE IN THE SMALL INTESTINE

BY
OVE LUNDGREN

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From the Department of Physiology University of Göteborg Sweden

STUDIES ON
BLOOD FLOW DISTRIBUTION AND COUNTERCURRENT EXCHANGE IN THE SMALL INTESTINE

BY

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GÖTEBORG 1967

This summary is based on studies reported in the following papers

I On the components of the Kr^{8} wash out curves from the intestine of the cat Kampp M O Lundgren and J Sjostrand Acta physiol scand 1967 In press

II Blood flow and flow distribution in the small intestine of the cat as analysed by the Kr^{8} wash out technique Kampp, M and O Lundgren Acta physiol scand 1967 In press

III Extravascular shunting of oxygen in the small intestine of the cat Kampp M, O Lundgren and N J Nilsson Acta physiol scand 1967 In press

IV The distribution of intravascularly administered lipid soluble and lipid insoluble substances in the mucosa and the submucosa of the small intestine of the cat Kampp, M, O Lundgren and J Sjostrand Acta physiol scand 1967 In press

The papers are referred to by their Roman numerals in the text

Introduction

Absorption secretion and motility constitute the three major functions of the small intestine These three functions are localized in different parts of the intestinal wall and have nutritional demands of different magni tudes Active absorption across the intestinal epithelium and secretion, which according to classical concepts occurs mainly in the crypts, are processes consuming considerable amounts of oxygen The motility of the muscularis on the other hand, requires comparatively little oxygen (*cf* Brodie and Vogt 1910 Martin and Fuhrman 1955)

It is well known that the magnitude of the blood supply to a tissue is closely adjusted to its current demand of oxygen and that regulatory mechanisms exist which increase blood flow to an organ in situations of enhanced metabolism However, in the case of the small intestine other functional aspects must also be of importance with respect to the proportions of its blood supply Thus blood flow of the intestinal villi is not only delivering oxygen, etc , needed for the absorptive work but also constitutes the major transport vehicle for absorbed material In a similar way the blood supply to glands does not only cover the nutritional demands of their work, but blood plasma also constitutes the raw material for the secretion In profusely secreting glands the need for fluid delivery can be high, indeed For such reasons it seems likely that the intestinal mucosa is often somewhat overperfused in relation to its nutritional oxygen demands (compare *e g* the kidney)

Although the aforementioned facts have been common knowledge for decades, most investigators studying the blood supply to the small intestine have recorded only its total blood flow However a change of total intestinal blood flow may not be homogenously distributed to the different intestinal wall layers but predominantly affect *e g* the circulation of the villi Nor may the same total blood flow to the intestine always reflect identical patterns of flow distribution in the intestinal wall A striking example of this was reported by Folkow and co workers (1964 a b) By means of a plethysmographic technique these investigators were able to show that stimulation of the sympathetic vasoconstrictor fibres to the small intestine diverted blood flow from the mucosa to submucosal vas cular pathways under conditions when total intestinal blood flow was

often only little if at all, decreased Similarly Jacobson, Swan and Grossman (1967) have demonstrated that gastrin and histamine can increase gastric secretion and gastric mucosal blood flow without significantly changing total blood flow of the stomach

In a few investigations, attempts were made to study the distribution of flow to the different layers of the intestinal wall utilizing in most cases lipid insoluble tracer substances such as deuterium oxide (DHO) or radio active rubidium (Rb^{86}) which were injected intra arterially or intravenously (Grim and Lindseth 1958 Rayner, McLean and Grim 1960, Weiner 1961, Csernay Wolf and Varro 1965 Weiner and Grim 1966) The amount of tracer present in the different layers of the intestinal wall were then determined It has been shown however that the fraction of the capillary surface area that is available for exchange of lipid insoluble substances is comparatively small (less than 1 per cent of total capillary area in skeletal muscle, Pappenheimer 1953) Experimental results indicate that the extraction of lipid insoluble solutes is dependent on the linear flow velocity of blood in the capillaries (*cf* Nolting *et al* 1958 Renkin 1959, Lassen 1964, Dresel Folkow and Wallentin 1966) There are consequently strong reasons to believe that the extraction of lipophobic material is not complete on one capillary passage except when blood flow is very slow It is possible at least theoretically to imagine two vascular beds with identical blood flows as expressed per unit weight tissue but with different mean transit times of blood in the capillaries open to flow and hence, different extractions of lipid insoluble substances

The transcapillary exchange of lipid soluble materials on the other hand, occurs across the entire capillary wall On theoretical grounds the use of inert lipid soluble gases, such as hydrogen, krypton or xenon seems therefore more satisfactory when studying regional blood flow In several tissues it has also been possible to demonstrate that the elimination of such easily diffusable inert gases is indeed, flow limited (for ref, see paper I)

The present series of experiments were started in an attempt to study the blood flow distribution in the small intestine of the cat by analysing the wash out curve registered by a scintillation detector after an intra arterial injection of krypton^{85} The recorded arched elimination curve could be resolved into four exponential functions Paper I deals with the localization of the different components of the γ curve as ascertained by four independent methods (registration of β activity, local tracer injections autoradiography weight comparisons) Knowing the localization of the various components in the intestinal wall it was possible to study in detail

the blood flow and its distribution within the small intestine Such an analysis was performed at resting blood flow and during various levels of vasodilatation as induced by graded infusions of a potent vasodilator drug isopropylnoradrenaline (II)

During the course of the experiments several observations suggested that the desaturation curve of Kr^{85} registered by the scintillation detector, did not exclusively reflect blood flow during low or moderate levels of total intestinal blood flow but perhaps also extravascular events Thus a large very rapid component of the γ curve was recorded during resting conditions Further an autoradiographic study revealed that a comparatively small amount of intra arterially injected lipid soluble antipyrine C^{14} reached the villi The vascular arrangement in the mucosa and particularly in the villi suggested the possible existence of a countercurrent exchange of easily diffusible materials in that part of the intestine Thus the small non branching arterial vessels ascending in the central parts of the villi are surrounded by a descending dense subepithelial capillary network (see below)

It was argued that if krypton was short circuited extravascularly between the two adjacent limbs of the mucosal vascular loops all easily diffusible substances including oxygen should act in a similar manner A study was therefore made to investigate the possible existence of an extravascular shunting of oxygen in the small intestine (III) This was done by comparing venous appearance time of oxygen with that of labelled red cells after close intra arterial administration

The presence of a mucosal countercurrent exchanger would affect the distribution in the mucosa of intravascularly injected substances The extent of diffusion exchange in a countercurrent exchanger is among other things dependent upon the capillary diffusion characteristics of the substance and on the linear velocity of blood Intra arterially injected lipid soluble material of small molecular weight passing through the hairpin vascular loop at a slow flow velocity will to a comparatively large extent be shunted extravascularly between the ascending and descending limbs of the vascular loops Thus only a small portion will reach the tissue supplied by the loop In order to obtain further indirect evidence for the existence of a mucosal countercurrent exchanger, a study was undertaken in which the distribution of lipid oluble and lipid insoluble substances in the mucosa and in the submucosa was investigated at various levels of blood flow (IV)

Preliminary reports of sections of this series of experiment have been published previously (Kampp and Lundgren 1966 a b Kampp Lundgren and Nilsson 1967 Lundgren and Kampp 1966)

Methodological Considerations

Observations were carried out on 76 cats anesthetized with chloralose i v (40—70 mg/kg) The cats had been deprived of food for at least 24 hours and had no obvious signs of intestinal infection The individual methods have been described fully in papers I—IV, which should be consulted for details A general survey is given below

A Operative procedures The operative procedures were identical in papers I—IV except for minor details The experiments were performed on intestinal segments weighing 20—50 g (mainly jejunum), prepared free from adjacent intestinal tissue The mesenteric vein which in such a preparation drains all the blood from the intestinal segment and its lymph nodes was cannulated and connected to a drop recorder unit operating an ordinate writer, recording on smoked kymograph paper The blood was returned to the animal via a funnel connected to the jugular vein Mean arterial blood pressure was recorded from the left femoral artery by means of a mercury manometer A small branch of the superior mesenteric artery was cannulated with a thin polyethylene catheter to permit close intra-arterial injections of isotopes to the intestinal preparation Close intra arterial infusions were made through a catheter inserted in a retrograde fashion into the superior mesenteric artery Intravenous infusions were given via a catheter in the left femoral vein The influence of the auto nomic nervous system was eliminated in most experiments by giving atropine i v (1 mg/kg) cutting the splanchnic nerves bilaterally and by denervating one adrenal gland and excluding the other from the blood circulation by ligatures

During radioactive measurements the intestinal segments were placed outside the abdomen on a wooden plate to ensure constant geometrical conditions The temperature of the intestine was continuously controlled with a thermocouple thermometer placed in the lumen of the gut Most of the serosal surface of the intestine and its mesentery was covered by Mylar® (DuPont) to hinder the diffusion of the radioactive gas from tissue to air Parts not covered by Mylar were protected by gauze soaked with bodywarm saline

B Intra arterial injections of Kr^{85} (I II) A saline solution containing krypton^{85} was given as a close intra arterial injection lasting 1—2 sec

The γ emission of Kr^{85} was recorded by an external scintillation detector placed 4—6 cm from the intestine The scintillation detector was coupled to a spectrometer and a linear ratemeter operating one channel of a three channel ink writer In order to ensure that only radioactivity from the intestine and its mesentery was registered by the scintillation detector the whole abdomen except the intestinal preparation was covered by a lead plate 5 mm thick Further a lead plate 5 or 10 mm thick was placed between the thorax and the detector Contamination of the air with Kr^{8} in the room was reduced to a minimum by a continuous suction of air around the funnel in the jugular vein as well as the animals expired air into a hood

The wash out of Kr^{8} was also recorded by two Geiger Muller tubes (I) registering the β emission of the tracer One tube was located at the antimesenteric border (below called external G M tube) mainly registering activity from the serosa and the muscularis The other tube was located in the intestinal lumen (below called internal G M tube) registering the β activity that mainly emanated from Kr^{85} in the villi and in the intestinal lumen The G M tubes were coupled to linear ratemeters operating two channels of a three channel ink writer

C *Local injections of* Kr^{85} *(I)* Local injections of minor amounts of a Kr^{8} solution were made into the different layers of the intestinal wall and into the perivascular fat of the mesentery When the tracer was injected into the mucosa the intestine was cut open along its antimesenteric border and mounted on a metal frame to prevent bleedings from the cut edges The elimination of the tracer was registered by a G M tube coupled to a linear ratemeter and an ink writer

D *Intravascular infusions of 4 iodoantipyrine and rubidium (IV)* Solutions containing the lipid soluble tracer 4 iodoantipyrine (labelled with $I^{1\ 5}$ or I^{131}) and the lipid insoluble $rubidium^{86}$ were infused intra arterially or intravenously After 20—45 sec from the start of the infusion 2—4 intestinal segments were extirpated and immediately frozen in an aceton dry ice mixture The villi were cut in three or four transverse sections by a freezing microtome The relative amount of the tracers in the sections was determined by means of a well type scintillation detector

E *Autoradiographic technique (I IV)* The distribution in the intestinal wall of urea C^{14} and antipyrine C^{14} was investigated autoradiographically The tracers were administered into the superior mesenteric artery as slug injections At a predetermined time after the injection a section of the small intestine was extirpated and frozen in an aceton dry-ice mixture The segment was sliced into thin transverse sections (5 10 or 20 μm) in a cryostat

The sections were mounted on glass and heated. They were then placed in close contact with X-ray film and exposed together with a drying agent at room temperature. The localization of the tracers in the intestine, as indicated by the blackening of the autoradiographs, was determined by simultaneous microscopical examination of the histological sections and their corresponding autoradiographs. In addition, a densitometric determination of the blackness of the autoradiographs was performed.

F. *Oximetric technique (III).* Light absorption of the venous effluent from the intestine was recorded continuously by means of a cuvette densitometer. The blood was drawn at a constant rate through a thin polyethylene catheter branching off from the venous outflow of the intestinal segment. The difference between the outputs of two photocells registering at wavelengths 625 and 805 nm, was recorded on an ink-writer.

G. *Blood substitute.* Low molecular weight dextran (10 per cent solution Rheomacrodex, Pharmacia) and dextran (6 per cent solution, Macrodex Pharmacia) mixed in a 1 : 2 relation was used to compensate for occasional blood losses and for the necessary filling of the blood flow system before its connection with the animals.

Theoretical considerations concerning the tracer elimination technique

The theoretical considerations outlined below are largely based on Kety's approach to tissue blood exchange and the reader is referred to his reviews for detailed discussions (Kety 1951, 1960). The general principles of the wash out method were reviewed at some length in papers I and II.

Provided that the arterial concentration of krypton is zero or negligibly low and that blood flow is constant during the registration period, the elimination of intra arterially injected Kr^{85} from the intestine as registered by an external scintillation detector, can be described by the following equation:

$$A_t = A_I \times e^{-k_I t} + A_{II} \times e^{-k_{II} t} + A_{III} \times e^{-k_{III} t} + A_{IV} \times e^{-k_{IV} t} \qquad (1)$$

(A_t = total counts per min at time t, A_I, A_{II}, A_{III}, A_{IV} = the number of counts per min initially present in each component, k_I, k_{II}, k_{III}, k_{IV} = clearance constants of the different components). The k- and A-values of the four components can be determined by peeling off exponentials. When performing such an analysis a straight line is drawn through the terminal straight (uniexponential[1]) portion of the curve and extrapolated

[1]) The terms "uniexponential" and "multiexponential" both of Latin origin have been used throughout the present study. The corresponding Greek words are "monoexponential" and "polyexponential".

to time zero This line is then subtracted from the original curve and a line is again drawn through the terminal straight part of the constructed curve The proceduce is repeated until a final uniexponential line is obtained The k values are determined from the slope of the components *i e* they may be determined from the half time values of the slopes (I) The A values are obtained from the extrapolation of each component to time zero

The abovementioned assumption concerning the negligible arterial concentration of krypton has been experimentally verified on man by Chidsay *et al* (1959) who found that less than five per cent of intravenously injected Kr^{85} appeared on the arterial side In the present study there are reasons to believe that the recirculation of Kr^{8} was if anything less than that observed by Chidsay *et al* since the blood after passing the drop chamber was allowed to drip through air into a funnel During this passage of blood through air a certain amount of Kr^{85} presumably evaporated from the blood The funnel was connected to a cannula in the jugular vein via which blood was returned to the animal A continuous suction into a hood was arranged around the funnel

A constant venous outflow during an experimental run as continuously recorded by the drop recorder unit was taken to indicate that the flow pattern in the intestine remained unaltered Experimental runs during which total intestinal blood flow changed significantly were discarded

The terms of equation (1) represents the wash out of krypton by blood provided that the tracer cannot leave the tissue by any other route than by the blood stream For that reason most parts of the intestinal segment were protected by Mylar® which prevents krypton from diffusing into the air Further the volume flow of intestinal lymph only amounts to 1/500—1/1000 of that of blood (Wilson 1962 page 9) a fact which excludes that any significant amount of the diffusible tracer leaved the intestine via that route In addition when stopping flow by simultaneously clamping the mesenteric artery and vein the desaturation of Kr^{85} γ activity ceased until blood flow was again restored

If it can be assumed that the equilibrium of the tracer between blood and tissue is extremely rapid *i e* negligible concentration gradients exist in the tissue blood flow (f) in a tissue or a tissue compartment can be calculated in ml/min × 100 g tissue from the formula

$$f = k \times s \times 100 \qquad (2)$$

Here k denotes the clearance constant [see equation (1)] and s denotes the tissue-blood partition coefficient of the tracer divided by the specific weight of the tissue Experimental data validating the assumption of a

blood flow limited wash out of krypton from the cat intestine, at least in the upper flow range was presented in paper II

In the case of a fast (instantaneous) intra arterial injection of the tracer, the relative distribution of flow to the different compartments of a multiexponential curve is estimated in the following way The amount of tracer present in each compartment at the moment of injection is determined by extrapolating each curve component to time zero (see above) By relating this amount to the initial total amount of the tracer the relative flow of blood to each compartment can be calculated It is assumed when doing these calculations that each compartment is registered by the detector with equal efficiency This assumption seems to hold true in the intestinal wall which is a symmetrical organ

It should be stressed that any wash out curve which on semilogarithmic paper has a general appearance similar to that registered in the present study (see upper panels of Fig 1 and 2 in paper I) can be resolved into a number of exponential functions and, consequently described in a manner similar to that of equation (1) The mere fact that such a component analysis can be performed does not necessarily imply that the different terms have any physiological significance in the sense that they describe the blood wash out of the tracer from particular parts of a tissue One may, for example mainly categorize a continuum of k values by such an analysis (*cf* Dobson and Warner 1957 Briscoe and Cournand 1959 Van Liew 1962, Kjellmer *et al* 1967)

In an attempt to establish whether the four components of the intestinal γ-curve were localized to particular parts of the intestinal preparation the registration of the elimination of γ activity was complemented by four independent methods (I) 1 The elimination of the Kr^{85} β activity was registered by two G M tubes (external and internal G M tube see above) 2 Minute amounts of a Kr^{85} solution were injected into different parts of the intestinal preparation and the elimination of the tracer was recorded by a G M tube 3 The wash out of antipryine C^{14} was investigated by means of autoradiography 4 The calculated relative weights of the two *slowest compartments of the γ curve were compared* with the measured relative weights of the muscularis and the mesentery The different techniques were consistent as to the localization of the different components For a detailed discussion of these matters the reader is referred to paper I here only the conclusion will be summarized briefly

The most rapid component of the intestinal γ curve predominantly reflected a countercurrent exchange of krypton between the ascending and descending limbs of the vascular loops in the mucosa, when the blood flow

was low or moderate During vasodilatation on the other hand it mainly mirrored the wash out of the tracer from a particularly well perfused part situated in the submucosa and adjacent parts of the mucosa The second and third components mainly reflected the disappearance of Kr^{85} from the mucosa and the muscularis, respectively The slowest component probably mirrored an elimination of Kr^{8} from the perivascular fat of the mesentery and an absorption of the tracer from the intestinal lumen

Selkurt and Wathen (1967) recently reported that the elimination curve of intra arterially injected $xenon^{133}$ from the intestine of the dog could be resolved into three components However these authors only registered the tracer wash out for 20 min, which might explain the differences in results when compared with those of the present study On largely indirect evidence Selkurt and Wathen suggested that the three intestinal components are localized in the glandular epithelium, the smooth muscle and the connective tissue Since they do not present any data corresponding to those reported in paper I any comparison of results is hardly possible

Zierler (1965 a b) has criticized the treatment of wash out curves as single exponentials or as a sum of exponentials Zierler s critique is based on a failure to record uniexponential elimination curves after intra arterial injections to the brain and the myocardium and after intramuscular injections (Zierler 1965 a) Brain (*cf* Sokoloff 1961 Haggendal Nilsson and Norback 1965) and in some situations also skeletal muscle (*cf* Kjellmer *et al* 1967) are however, known to be heterogenously perfused by blood which explains the shape of the wash out curve Other investigators have shown the desaturation curve from the myocardium to be uniexponential when recording the elimination of $krypton^{85}$ by means of a scintillation detector (Johansson Linder and Seeman 1964) or the wash out of hydrogen by a polarographic method (Aukland Bower and Berliner 1964) In the latter study a close similarity was noted between the blood flow value deduced from the tracer wash out and the measured venous outflow Furthermore Setchell Waites and Thorburn (1966) recently demonstrated that the elimination of krypton from the testis of the ram showed a uni exponential decline even when the registration was prolonged until an activity level of only 1—2 per cent of the initial was reached Thus the equations developed by Kety appropriately described the wash out of the tracer from the myocardium and the testis Although one cannot exclude the possibility that Zierler s criticism is valid for some tissue the afore mentioned results exclude a general applicability of his critique

The problem is more complex as far as the intestine is concerned The multicompartmental nature of the krypton elimination curve from this

tissue as registered by the scintillation detector, was *a priori* expected since the gut contains layers with different functions and metabolic demands (see above) and hence in all probability different blood flows Therefore the registration of a multiexponential intestinal wash out curve may well reflect a simultaneous recording of several uniexponential decays This opinion is corroborated by the uniexponential wash out curve usually registered in the present study by the external G M tube Further, this elimination curve exhibited a k-value as great as the third component of the γ curve In some experiments, however an initial rapid component was registered by the external β curve In experiments performed during resting blood flow, the k value of the first component agreed very well with the second component of the γ curve in others performed during vasodilatation the k value corresponded with the first component of the γ-curve Furthermore uniexponential elimination curves were recorded after local injections into the mucosa submucosa, the muscularis serosa and the perivascular fat of the mesentery the slopes of which were as great as that of the second third and fourth components of the γ curve respectively Finally, the autoradiographic investigation clearly indicated that the wash out was not equally fast from all parts of the intestinal wall In conclusion, it may be stated that all experimental data, described above, strongly suggest that the intestinal γ curves were composed of four fairly distinct families of k values localized in particular parts of the small intestine

Blood flow and flow distribution in the small intestine

Anatomical considerations

The vascular anatomy of the intestinal tract has been extensively studied in various animals (for reviews of the literature see Hou-Jensen 1930 Spanner 1932 Patzelt 1936, Wiedeman 1963) The following description presents the major traits of the small intestinal vasculature in mammals

The small intestine has been found to be composed of a number of parallel coupled vascular beds The arteries pierce the intestinal muscularis obliquely and form an extensive vascular plexus in the submucosal layer The intestinal muscularis is supplied by blood via vessels branching off from the arteries before and during their course through the muscularis and from the submucosal plexus The mucosal arteries also emanate from the submucosal vascular network Separate vessels seem to supply the crypts and the villi (Heller 1872 Mall 1888 Patzelt 1936) The details of the vascular arrangement in the villi will be described in the next chapter The course of the intestinal veins is largely similar to that of the arteries

A characteristic vascular structure is present in the submucosa of certain animals It consists of arterioles containing longitudinal smooth muscles which arborize into a dense meshwork of thin walled veins These structures were initially described in the dog by Mall (1888) and later in other animals including the cat by Spanner (1932) In the cat these so-called Venenballchen are localized in two parts of the submucosa One group is situated in close connection to the circular layer of the muscularis [illegible] the other is located between the larger vessels of the submucosal plexus According to Spanner (1932) there are 170 Venenballchen per [illegible] the submucosa of the cat In man it has not been possible to demo[illegible] any corresponding vascular structures

Results

The blood flow distribution of the acutely denervated [illegible] was studied at resting blood flow levels and during vasodila[illegible] by graded constant intra arterial or intravenous infusions of [illegible] adrenaline (II) The resting total intestinal blood flow a[illegible]

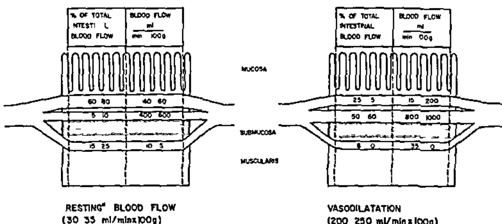


Fig 1

The flow distribution of the small intestine of the cat at resting total intestinal blood flow (left section) and during intense vasodilatation (right section) The middle vessel of the two sections depicts a well vascularized area localized in the submucosa and adjacent parts of the mucosa

50 ml/min × 100 g a value somewhat less than that reported for the cat by Folkow, Lundgren and Wallentin (1963) The observed discrepancy may possibly be ascribed to the fact that the animals of the present study had fasted for a longer time than those used in the study of Folkow, Lund gren and Wallentin When vasodilatation was induced by means of iso propylnoradrenaline flows up to 225 ml/min × 100 g were recorded

The pattern of blood flow distribution varied to a considerable extent at low and high venous outflow as is illustrated in Fig 1 At 'resting levels of blood flow (30—35 ml/min × 100 g tissue, see left panel of Fig 1) most of the intestinal blood flow (60—80 per cent) was diverted to the mucosa to give an average blood supply to this section of 40—60 ml/min × 100 g Only 15—25 per cent of the total blood flow was delivered to the muscularis corresponding to a blood supply of 10—15 ml/min × 100 g of this tissue 5—10 per cent of the total blood flow was diverted to a small but well perfused tissue section located in the submucosa and in the immediate surroundings of the intestinal crypts where the average blood flow was measured at 400—600 ml/min × 100 g It should be pointed out that the values given on blood flow and flow distribution of this well perfused part are approximate (II)

During vasodilatation produced by isopropylnoradrenaline the intestinal blood flow pattern became markedly altered (see right part of Fig 1) As much as 50—60 per cent of the total flow was distributed to the very well perfused tissue section in the submucosa and around the crypts which had now greatly increased in size where blood flow levels as high as 800—1000 ml/min $\times$ 100 g were then measured About 25—35 per cent of total intestinal blood flow was delivered to the mucosal vascular bed giving here an average flow level of 150—200 ml/min $\times$ 100 g Slightly less than ten per cent of the total flow was diverted to the muscularis corresponding to 35—40 ml/min $\times$ 100 g of this tissue

From Fig 1 and the description above however, it is not evident that in addition sections of the mucosa and the submucosa seemed to exist where in all probability the blood flow rate was largely similar to that of the muscularis (I, II) The amount of blood flow distributed to these less well-perfused sections of the mucosa submucosa was five per cent of the total intestinal flow at most Furthermore in Fig 1 and the description above the minor fraction of intestinal blood flow diverted to the perivascular fat of the mesentery (I II) has not been considered

Discussion

The small but highly vascularized section of the intestinal wall located in the submucosa and the immediate surroundings of the mucosal crypts hardly corresponds to any true vascular shunt arrangements but is thought to contain vascular sections that allow a free exchange of at least lipid soluble substances across their walls This opinion is based on the fact that it was possible to localize the well perfused region in the autoradiographic study using antipyrine C^{14} (I IV) Had the blood here exclusively been diverted through arterio venous shunt vessels, not allowing any exchange across their walls it would hardly have been possible to demonstrate this well perfused tissue section by the present autoradiographic technique The functional significance of this extremely well perfused but minor section of the intestinal wall is not yet quite clear Some possible alternatives will be discussed below

The appearance of the Venenballchen in the submucosa as described above suggests in itself that they receive a high blood flow rate Further more this thin walled delicate network of minor vein like vessels may, in analogy to other venular sections well allow for exchange across their walls at least of lipid soluble substances It therefore seems probable that the highly vascularized tissue section located in the submucosa and the

surroundings of the crypts (see below) corresponds in part to the submucosal Venenballchen A gradually increasing weight of the first compartment of the γ curve was noted when flow was raised by infusions of increasing amounts of isopropylnoradrenaline (II) This observation may indicate, that progressively larger numbers of these peculiar vascular structures were opened for flow by vasodilatation A *redistribution of* intestinal blood flow to arterio venous pathways also appears to occur in the dog when intestinal vasodilatation is induced by acetylcholine to judge from indirect evidence presented by Varro *et al* (1967 a) On the basis of his morphological findings Spanner (1932) proposed that intestinal blood flow may become diverted through the submucosal Venenballchen during *e g* hunger thus augmenting the oxygen delivery via the portal blood to the liver He also suggested that portal venous pressure increases upon opening of these vascular structures securing a proper perfusion pressure for the liver

It was proposed by Folkow and co workers that flow is diverted through the Venenballchen during sympathetic vasoconstrictor activity (Cobbold *et al* 1964, Folkow *et al* 1964 b, Dresel, Folkow and Wallentin 1966 Dresel and Wallentin 1966 Wallentin 1966) If so it would imply that some section of the vascular walls of these characteristic structures would allow for a largely free exchange also of lipid insoluble substances between blood and tissue , since there was no evidence that any significant part of the blood stream was shunted during this neurogenic redistribution (Dresel Folkow and Wallentin 1966) Such a redistribution would guarantee the liver enough blood and oxygen supply during even profound activities of the sympathico adrenal system in *e g* alarm reactions (Cobbold *et al* 1964) It was furthermore suggested by these authors that such a redistribution of intestinal blood flow may suppress the secretory activity of the intestinal glands simply by depriving them of the blood supply necessary for the secretory work

The autoradiographic study (I, IV) indicated that the aforementioned well perfused part of the intestinal wall to some extent was also located in the tissue between the mucosal crypts No Venenballchen have been observed in that part of the intestine (*cf* Spanner 1932) The function of the intestinal crypts suggest however other reasons for a rich blood supply to this particular section of the intestinal mucosa It has now been firmly established that the turnover rate of the intestinal epithelium is extremely rapid (for ref see Creamer 1965) The total population of epithelial cells in the cat duodenum or ileum is thought, for example to be replaced in 2—3 days It has been estimated that about 250 g of cells are released daily

into the intestinal lumen in man (Wilson 1962) The production of new epithelial cells takes place in the crypts, and the newly formed cells apparently move from the crypts up along the villi simply by gliding over the basement membrane (Creamer 1965) This rapid cell renewal occurring in the crypts presumably constitutes an energy consuming process that requires a large supply of oxygen and hence of blood flow

Further, according to the classical views on intestinal function the secretory activity of the small intestine occurs in the mucosal crypts Secretion is known to call for a large supply of oxygen It has for example been demonstrated that the oxygen consumption during maximal secretory work of the dog salivary gland amounts to 7—8 ml per hour and per g tissue (Terroux Sekelj and Burgen 1959), *i e* an oxygen consumption approximately 30 times greater than that observed in resting cat skeletal muscle (Quensel and Kramer 1939) In addition the blood flow to a secretory organ also has to furnish the raw material for the secretion in terms of water and solutes During maximal secretory activity blood flow of *e g* the cat salivary glands amounts to more than 500 ml/min × 100 g (Martinson and Odelram unpublished observations) Such a high blood flow is needed to provide enough fluid for secretion without too greatly increasing the viscosity of the passing blood The blood flow of the secretory parts of the small intestine is, therefore also for such reasons large particularly if the secretion rate per unit tissue is high

In summary, it is proposed that the well perfused part of the intestinal wall is localized partly to the submucosal Venenbällchen and partly to the mucosal crypts concerned with epithelial cell renewal and secretion The amount of blood flow distributed to each of these regions cannot be determined by the present technique

The blood flow of the mucosa and particularly of the villi is of paramount importance for the absorption taking place across the intestinal epithelium Blood flow not only delivers the oxygen needed for the absorption work but also constitutes the transport vehicle for absorbed material In the present study mucosal flow was increased from about 40 ml/min × 100 g at rest up to 200 ml/min × 100 g during intense vasodilatation induced by infusion of isopropylnoradrenaline An increase in total intestinal or splanchnic blood flow during digestive work has been repeatedly observed (Brodie Cullis and Halliburton 1910 Brodie and Vogt 1910 Herrick *et al* 1934 Lowenthal Harpuder and Blatt 1952 Brandt *et al* 1955 Reininger and Sapirstein 1957 Grim and Lindseth 1958, Bursadoun and Reid 1962 Varro *et al* 1967 b) concomitant with an increased oxygen consumption in the splanchnic area (Brodie Cullis and

Halliburton 1910, Brodie and Vogt 1910 Smythe, Fitzpatrick and Blakemore 1951, Varro *et al* 1967 b) The blood flow increase observed by the aforementioned authors amounted to 150—200 per cent above control, at most, but usually the augmentation was less than 100 per cent Thus the average increase in regional blood flow during digestion was considerably less pronounced than the maximum increase of mucosal flow observed in the present study The discrepancy may be explained in two ways First, in most of the aforementioned studies, total intestinal flow was usually measured and it seems unlikely that the entire digestive tract should become simultaneously vasodilated to the same extent during digestion Second the increase of blood flow in the intestinal wall may be mainly confined to the mucosal section although some flow augmentation probably also occurs in the muscularis due to its increased motility It should be underlined however, that the absorption of certain food may not induce any drastic changes in intestinal blood flow (*cf* Brandt *et al* 1955)

In the present experiments the blood supply to the muscularis was also *enhanced during infusion of isopropylnoradrenaline from a resting value* of 10—15 up to 35—40 ml/min × 100 g muscle tissue at intense vasodilatation This flow range of intestinal smooth muscle is largely similar to that observed in the cat skeletal muscle (Cobbold *et al* 1963) Furthermore, the observed flow values of the muscularis agree well with those of other smooth muscle organs, *e g* the uterus (Reynolds 1963, Munck *et al* 1964, Lysgaard and Lefevre 1965) or the cat bladder (Folkow Lisander and Lundgren unpublished observations) Intestinal motility induced by stimulation of the vagal nerves, does not seem to increase total intestinal blood flow significantly (Kewenter 1965, Lundgren unpublished observations) nor increase the intestinal capillary filtration coefficient (Lundgren unpublished observations) Although muscularis blood flow is comparatively small, a 2—3 fold increase during motility should have inducted an easily discernible increase of total intestinal flow It may, therefore be questioned whether the full range of muscularis blood flow is ever fully utilized during physiological circumstances

Countercurrent exchange in the small intestine

A large very rapid component of the Kr^{85} wash out curve as registered by the external scintillation detector was observed at resting total intestinal blood flow (I II) and suggested a considerable shunting of the tracer within some sections of the intestinal wall even at low or moderate levels of total blood flow It seemed *a priori* unlikely however, that as much as 30—50 per cent of total intestinal blood flow should be diverted through arterio venous blood flow shunts at 'rest Furthermore Grim and Lindseth (1958) utilizing radioactively labelled glass spheres had estimated the shunt blood flow (A V anastomotic flow) of the dog intestine to only 3—4 per cent of total intestinal flow Thus other explanations had to be sought for

Two technical artefacts were considered as possible explanations Firstly, a rapid diffusion of the tracer from the tissue to air and away from the detector might explain the experimental findings However, in the present experiments the intestinal tissue was covered by Mylar and gauze soaked in body warm saline (I) hindering such a fast diffusion Secondly it might be argued that the first component of the γ curve might simply represent an injection artefact at low intestinal blood flow However such an interpretation was made less plausible by the finding that the desaturation curve from the stomach registered after an intra arterial injection of a Kr^{85} solution did not show any corresponding initial, rapid component (Jansson *et al* 1966)

Desaturation curves of inert gases registered from the brain (Haggendal Nilsson and Norback 1965) from the kidney (Aukland Bower and Berliner 1964) and from the skeletal muscle (Aukland Akre and Leraand 1967) have been reported to exhibit fast initial components, not believed to reflect blood flow The initial component of the Kr^{85} wash-out curve registered by a scintillation detector from the brain was thought to constitute the activity of the tracer in the arterial blood About 15—20 per cent of total radioactivity appeared to be distributed to such a component at the time of the injection and the first component was easily registered by a G M tube placed above the brain surface The failure to register the corresponding component of the intestinal γ curve by the external and internal G M tubes at resting blood flow indicates that the radioactivity

in the arterial blood cannot contribute to any significant extent to the first component of the intestinal γ curve The absence of any corresponding component in the Kr^{85} desaturation curve from the stomach further strengthens this conclusion

The 'shunting of hydrogen registered by a catheter hydrogen electrode located in the renal vein after an intra arterial injection of hydrogen saturated saline was believed to reflect a countercurrent exchange of the gas between the two limbs of the vasa recta loops (Aukland 1964) A countercurrent exchange of the hydrogen was also thought to be mirrored in the fast component of the skeletal muscle elimination curve registered by a catheter hydrogen electrode in the femoral vein (Aukland, Akre and Leraand 1967) Since the vascular arrangement of the intestinal mucosa particularly in the villi (see below), is in accordance with a similar extra vascular gas shunt, experiments were performed to test this possibility

Anatomical considerations

The vascular arrangement of the intestinal villi varies among mammals (*cf* Heller 1872 Spanner 1932, Jacobson and Noer 1952) The following description presents the details of the vascular architecture of the villi in cat and dog The villous anatomy of these two species is largely similar (Heller 1872 Mall 1888, Nisioka 1927, Jacobson and Noer 1952)

The arterial supply to each villus consist of a single tortuous vessel running in the central part of the villus without branching (see Fig 2) The diameter of this vessel is approximately 20 μm It loses its muscular coat during its course in the villus (Mall 1888) The possible existence of pores in the endothelial lining of this central arterial vessel does not seem to have been investigated Close to the tips of the villus this ascending vessel arborizes into a dense subepithelial network of capillaries The presence of endothelial fenestrations with a diameter of approximately 500 Å covered by a thin basement membrane and of fissures between endothelial cells 100—150 Å wide have been demonstrated in these capillaries by electron microscopy (Bennet Luft and Hampton 1959, Palay and Karlin 1959, Horstman 1966) The fenestrated part of the capillary walls seems to face the epithelial cells (Horstman 1966) In the cat the capillaries collect into veins at the villous base (Heller 1888 Nisioka 1927) while some investigators claim that in the dog the villous vein already begins to be formed close to the tip of the villus (Mall 1888, Jacobson and Noer 1952)

It is clear that the *main* direction of blood flow in the subepithelial

BLOOD CIRCULATION IN VILLUS (CAT)

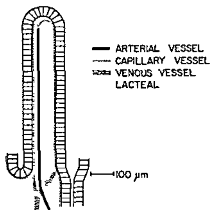


Fig 2

Schematic drawing of the vascular anatomy of a cat villus The figure is mainly based on the histological measurements of villi made on cats by Schriever (1899) The capillary vessels of the figure denote a dense capillary network lying just beneath the intestinal epithelium Note that the ascending arterial vessel and the descending capillary network and venous vessel form a hairpin vascular loop

capillary network must be opposite to that of the central arterial vessel (Fig 2) The distance between the two limbs of these vascular hairpin loops in the villi can be deduced from the data presented by Schriever (1899) In histological sections he estimated the distance between the central lacteal and the epithelial cells to be approximately 40 µm If the diameter of the arteriole and the capillaries are assumed to be 20 and 10 µm respectively, the shortest intervascular distance between the two limbs of the loop would amount to approximately 10 µm Correcting for a shrinkage during the histological preparation procedure amounting to 50 per cent of the dimensions of the histological sections the aforementioned intervascular distance would amount to 15—20 µm It should be pointed out however that the dimensions of the villi seem to vary in different functional states (*cf* Wells and Johnson *1934*)

No data seem to be published which make it possible to deduce the distance between the villous arterial and venous vessels in their course through the tissues between the crypts In any case the anatomical prerequisites for a countercurrent exchange *i e* the close association of the

arterial and venous limbs of a vascular loop, seem to exist at least in the intestinal villi In this connection it should be stressed that the vascular anatomy of other villous structures, such as the villi of the synovial tissue (Lindstrom 1963) differs from that of the intestinal villi and seems to constitute a less efficient countercurrent exchanger

Experimental evidence

1 In all experiments of the present study, in which the wash out of Kr^{8} was registered total venous outflow from the intestinal segment was continuously recorded by a drop recorder unit Total intestinal blood flow could also be calculated from the Kr^{85} wash out curves (II) and it was accordingly possible to compare the two methods of estimating flow Such a comparison revealed that only a small part of the first component of the γ-curve had to reflect blood flow to account for the recorded total intestinal blood flow when venous outflow was less than 60 ml/min × 100 g When total intestinal blood flow exceeded 60 ml/min × 100 g, an increasing portion of the initial component seemed to mirror true blood flow and when flow values above 150 ml/min × 100 g were recorded, the first component appeared to reflect blood flow exclusively (see Fig 3 in paper II) These observations alone could indicate that extravascular events such as a countercurrent exchange, constitute at least part of the initial rapid component of the γ curve at blood flow levels below approximately 150 ml/ min × 100 g

2 In an attempt to localize the different components of the γ curve, an autoradiographic study was performed utilizing antipyrine-C^{14} In these experiments intestinal segments were removed at various times after an intra arterial injection of the tracer at different levels of total intestinal blood flow Autoradiographs were made of 5 10 or 20 μm thin tissue slices It was demonstrated by this technique that the localization of the fastest rates of wash out, *i e* those corresponding to the first component of the Kr^{8} , curve seemed to move from the intestinal tissue around the crypts towards the tips of the villi when total intestinal blood flow was increased from 20 to 100 ml/min × 100 g by graded intra arterial infusions of isopropylnoradrenaline (Fig 4 in paper IV)

The results described above (points 1 and 2 of *Experimental evidence*) may be explained by the countercurrent hypothesis in the following way When resting intestinal blood flow prevails, the linear velocity of blood in the mucosa is comparatively low A relatively large fraction of the intra arterially injected tracer will be short circuited between the two limbs of the mucosal vascular loops in the tissue around the crypts When total

intestinal blood flow is increased by means of the vasodilator agent isopropylnoradrenaline mean transit time of blood through the mucosal vasculature will in all probability be shortened This assumption is based on the observations reported by Folkow, Lundgren and Wallentin (1963) that total intestinal blood volume increased approximately only 40 per cent above resting at maximal dilatation while the concurrent increase of total intestinal blood flow amounted to 400—500 per cent It is further clear from the Poiseuille law that a radius increase other things being equal will increase volume flow to the fourth power of this radius increase and the linear flow rate to the second power Although the countercurrent diffusion exchange of the lipid soluble tracers antipyrine C^{14} and krypton is presumably very fast it certainly takes a definite length of time Therefore as the linear velocity of mucosal blood flow is increased the main part of the countercurrent diffusion will take place in more and more distal parts of the intestinal vascular tree, *i e* it will tend to move from the tissue around the crypts towards the tips of the villi Concomitantly, the fraction of injected tracer shunted in the mucosal countercurrent exchanger will decrease Finally a blood flow level is reached (above 100—150 ml/min $\times$ 100 g) at which transit time becomes too short to allow any more significant short circuiting between the ascending and descending limbs of the mucosal vascular loops (II IV)

3 It was argued that if antipyrine and krypton were short circuited extravascularly between the two limbs of the mucosal vascular loops other easily diffusible substances should behave in a similar manner In order to obtain evidence for such a shunting of oxygen, the venous appearance time of intra arterially injected oxygen was compared with that of methemoglobinemic red cells administered in an identical manner Although red cells have been shown to pass more rapidly through the splanchnic vasculature than plasma (Chien 1963) it was found that oxygen appeared 1—2 sec earlier than the labelled red cells in the mesenteric vein during resting conditions This observation is explained by the countercurrent hypothesis in the following way Oxygen is shunted in the mucosal countercurrent exchanger and can therefore appear earlier in the mesenteric vein than the labelled red cells which remain within the vessel and consequently have to pass through the mucosal vascular loops

Results similar to those obtained in the present study on the intestine have been reported for the kidney (Levy and Sauceda 1959 Levy and Imperial 1961) The early appearance of oxygen in the renal vein has been taken to indicate a countercurrent exchange diffusion of oxygen between the limbs of the vasa recta loops

The results presented above under points 1—6 summarize the experimental evidence obtained in the present investigation for the existence of a countercurrent exchange in the intestinal mucosa of the cat Although alternative explanations can be suggested for each of the different observations a countercurrent exchange seems to be the only mechanism which easily and adequately explains *all* these experimental findings

It might be argued that the countercurrent exchange mainly occurs across the walls of the larger intestinal vessels, *e g* in the extensive submucosal network A countercurrent exchange diffusion of hydrogen has recently been reported to occur between evidently fairly large arterial and venous vessels in skeletal muscle (Aukland, Akre and Leerand 1967) Although it is not possible with the technique of the present study to exclude that such a countercurrent exchange to some extent also takes place *e g* in the intestinal submucosa, the experimental findings reported above under points 2, 4 5 and 6 clearly indicate the existence of a considerable countercurrent exchange between the vessels of the intestinal mucosa, particularly in the villi It should be pointed out in this connection that the Kr^{85} elimination curve, registered from the stomach lacks any component corresponding to the very rapid one of the intestinal γ curve (Janson *et al* 1966) The stomach has a submucosal vascular network similar to that of the intestinal wall but lacks the hairpin loop vascular arrangement of the mucosa (*cf* Babkin 1950) which thus appears to be necessary for an efficient countercurrent exchange

When the intestinal vascular bed was dilated by means of isopropyl noradrenaline it could be demonstrated that there was no indication of any significant countercurrent exchange above a certain flow level Thus, when total intestinal blood flow exceeded 60—70 ml/min × 100 g oxygen, in any detectable amount no longer appeared before labelled red cells in the venous effluent (*cf* point 3) At approximately the same blood flow level the time of delay as recorded by the internal G-M tube reached a constant level of about 0 5 min (see point 6) suggesting the absence of any significant countercurrent exchange of Kr^{85} The autoradiographic study however provided evidence that *a certain countercurrent exchange* of antipyrine-C^{14} still took place even when total intestinal blood flow amounted to 100 ml/min × 100 g while it was made probable that no significant exchange occurred when blood flow was 175 ml/min × 100 g (IV) These findings were corroborated by the analyses performed on the first component of the Kr^{85} γ curve (Fig 3 in paper II and point 1 above),

which indicated that some countercurrent exchange was still evident at blood flow levels as high as 150 ml/min × 100 g

The apparent discrepancy between the results described above obtained by the different techniques of the present study, may be explained in the following way The failure to demonstrate an earlier appearance of oxygen than of labelled red cells in the venous effluent during vasodilatation may be due to an opening up of arterio-venous short circuits (possibly the Venenballchen , see paper III) where the transit time was so short as to mask any simultaneously occurring extravascular shunting of oxygen Further, the spatial relationship betwen the villi and the internal G M tube may offer an explanation for the results obtained with this detector Thus it seems reasonable to assume that the internal G M tube was not only in contact with the tips of the easily bending villous structures, but also with their long sides If so a countercurrent exchange taking place *e g* in the middle part of the villi could hardly be differentiated from one occurring at the very tips The failure to demonstrate any fast wash out by the internal G-M tube may be explained by a diffusion of the tracer towards the internal G M tube masking any fast tracer wash out in the less closely situated countercurrent exchanger

In conclusion it seems reasonable to assume that the countercurrent exchange of lipid soluble solutes in the intestinal mucosa of the cat becomes relatively insignificant when intestinal blood flow exceeds 100—150 ml/min × 100 g The countercurrent exchange of lipid insoluble material seemed to become insignificant already when intestinal blood flow was approximately 100 ml/min × 100 g to judge from the results obtained with urea C^{14} (IV)

The vast literature on intestinal absorption (for reviews see the monographs of Verzar 1936 Wilson 1962 and Wieseman 1965) provides several observations that may be taken as support of the present countercurrent hypothesis The examples presented below, taken from the literature on the absorption of gases monosaccharides and water do not pretend to be exhaustive Further it should be stressed that these earlier studies were not aimed at exploring the existence of any mucosal countercurrent exchange or considered such to be a possibility Any control experiments to rule out other possibilities were obviously not performed

It seems *a priori* likely that the presence of a countercurrent exchanger in the intestinal mucosa must affect intestinal absorption in several ways The transport of a substance, active or passive , from the lumen across the intestinal epithelium into the subepithelial capillary network of the

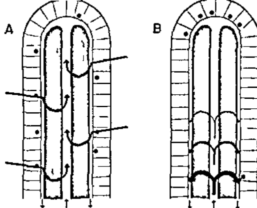


Fig 3

The functional implications of the mucosal countercurrent exchanger schematically illustrated The intervascular distance is greatly exaggerated for the sake of clarity For details see text

villus will increase the concentration of the solute in the venous blood draining the villus and consequently create an arterio venous concentration difference Because of the vascular anatomy of the villus, a fraction of the substance can then be expected to diffuse extravascularly to reach the central ascending arterial vessel, as schematically illustrated in section A of Fig 3 Thus, the solute is again brought towards the tip of the villus The effectiveness of the countercurrent exchanger to delay net absorption of substances in such a way must depend on factors such as the distance between the two limbs of the hairpin loop the permeability of their walls the linear velocity of blood in the vessel the diffusion characteristics of the solute etc

Evidence for a delayed absorption of the inert lipid soluble substances antipyrine and krypton during low and moderate blood flow levels within the intestinal tissues was presented in the present study (I, IV, see also Kampp and Lundgren 1966 b) These findings are corroborated by the observation in earlier studies that gaseous hydrogen, when introduced into the lumen of the small intestine is absorbed extremely slowly if at all (Kato 1910 Schoen 1923, McIver, Redfield and Benedict 1926)

The concentration of an absorbed easily diffusible substance in the venous effluent from the villus would, according to the countercurrent exchange hypothesis be comparatively small at low and moderate blood

flow levels as evident from Fig 3 A A large concentration difference would then be expected to exist between its concentration in the intestinal lumen and that in the venous effluent from the intestine Such a large con centration ratio between intestinal lumen and venous effluent from the intestine can also be calculated from experiments dealing with the absorption of glucose (30 1 to 50 1 Kotschneff 1923 Magee and Reid 1931) and DHO (15 1 to 25 1 Grim Lee and Visscher 1955 Winne 1966) It may, however, be argued that these large differences in concentration may be fully explained as well by other factors such as the relative im permeability of the intestinal epithelium to lipid insoluble substances (Wil son 1962) concentration differences in the intestinal lumen metabolic conversions of the solute during its passage across the intestinal epithelium the dilution of the absorbed substance in the mesenteric vein by blood not equilibrated with the content in the intestinal lumen etc

During the absorption of gaseous carbon dioxide a lipid soluble compound of small molecular weight all the above-mentioned factors with exception of the last mentioned one would be expected to be of little relevance Nevertheless the ratio between the carbon dioxide tension of the intestinal lumen and that of the blood in the superior mesenteric vein has been reported to be as high as 8 1 to 9 1 (Pals and Steggerda 1966) The dilution of CO in the venous effluent from the intestine by admixture of blood not equilibrated with the contents of the intestinal lumen may account for a concentration ratio of the order of 2—3 1 at most, to judge from the data on blood flow distribution in the intestinal wall obtained in the present study The findings of Pals and Steggerda are further corroborated by the high carbon dioxide tension of the intestinal contents reported by deBeer Johnston and Wilson (1935) Herrin (1937) and McGee and Hastings (1942)

The study of Grim Lee and Visscher (1955) referred to above calls for some further considerations In this study the intestinal lumen was lavaged for 0 5—120 min by a fluid containing an approximately constant con centration of D O It was found that the concentration of D O in the mucosa and in the venous effluent from the intestinal segment was sur prisingly low amounting at most to 25 and 5 per cent of that of the intes tinal lumen respectively The authors discuss at length possible explana tions for these observations and conclude that they may be explained by either a low mucosal capillary permittivity to water or an extensive arterio-venous shunt carrying at least 35 per cent of the total blood flow through the intestine In the light of the present investigation it seems likely that the aforementioned observations of the concentration of D O

in the intestinal mucosa and the mesenteric venous blood are caused by the presence of a mucosal countercurrent exchanger This opinion is corroborated by the finding that no significant shunting of blood seems to occur in the dog intestine (Grim and Lindseth 1958)

Examples like those mentioned above may thus suggest that the countercurrent mechanism is of functional importance for intestinal absorption but clearly calls for exploration by means of experiments that are specifically designed to test such a possibility It seems reasonable to assume that a delay of absorption will occur as a result of a countercurrent mechanism in the villi, thus forming an automatic damping device to hinder too *rapid intestinal absorption particularly of easily diffusible substances* Such a mechanism may for example, help to prevent sudden and large variations in the osmolarity of the blood leaving the small intestine thus protecting the liver and the rest of the organism from osmotic shocks The deleterious effects of the hyperosmotic solutes in the intestine are well known from the dumping syndrome in humans and may be at least partly related to the creation of hyperosmolarity in the blood

The existence of an intestinal countercurrent exchanger may affect not only intestinal absorption of materials but also, in certain situations substances that reach the exchanger via the arterial blood In the presence of a higher arterial than venous concentration of a solute the substance may be short circuited extravascularly and more or less excluded from the villus as shown in section B of Fig 3 Such a concentration difference between artery and vein may be induced in two ways *i e* by increasing the arterial concentration or by decreasing the venous concentration of the solute In the present series of experiments the arterial concentration was increased by intravascular administration of various solutes It was then demonstrated that antipyrine and 4 iodoantipyrine were more efficiently excluded from the villus than urea and rubidium a difference anticipated on grounds of their different lipid solubility (IV)

It is probable that an arterio venous concentration difference of blood borne solutes is often established in the intestine of the intact organism by a diffusion from the subepithelial capillary network into the intestinal lumen thus tending to reduce their concentration in the venous effluent The countercurrent exchange diffusion between the arterial and venous vessel of the villi prevents to some extent such a loss of solutes, from the blood into the lumen of the gut and establishes a continuous concentration decrease of the substance from the bases towards the tips of the villi The mechanism is selective in the sense that those substances that would most easily traverse the intestinal epithelium (lipid soluble material of small

molecular weight) will also be most efficiently shunted in the mucosal countercurrent exchanger

The mechanism illustrated in Fig 3 B will to some extent presumably hinder the net blood transport also of oxygen to the tips of the villi, at least at low and moderate flow levels and create a decreasing oxygen pressure within the villi from their bases towards their tips This assumption is corroborated by the experimental findings of paper III that strongly suggest the existence of an extravascular shunting of oxygen in the small intestine The proposed oxygen gradient in the villi may be a factor of importance in explaning the well known rapid turnover of intestinal epithelial cells (see preceeding chapter)

Several attempts have been made to determine the oxygen tension in the intestinal mucosa in birds and mammals including man and the observed values range between 20 and 40 mm Hg in most species (Schoen 1925 McIver Redfield and Benedict 1926, Campbell 1931 Roger 1949 Crompton Shrimpton and Silver 1965 Dawson Trenchard and Guz 1965) However the venous oxygen tension or the total intestinal blood flow was not determined in any of these studies Further present techniques will hardly allow an exact localization of the recorded oxygen tension in the mucosa e g with respect to basal or apical parts of the villi From what has been discussed above it is however, likely that the oxygen tension may be markedly different in different parts of the villi It therefore seems impossible on the basis of available data concerning regional oxygen tension to assess whether the difference in oxygen tension between the intestinal mucosa and the blood of the mesenteric vein expected from the countercurrent principle really is present

It has on the other hand been observed that a reduction of intestinal blood flow brought about by graded mechanical clamping of the intestinal artery decreases intestinal oxygen consumption (Varro *et al* 1965 Baker and Mendel 1967) This observation may at least in part be explained by a gradually increasing shunting of oxygen in the mucosal countercurrent exchanger when the flow velocity of blood in the mucosal vascular loops decreases Less oxygen can then reach the apical parts of the villi to be consumed there Such a mechanism may have an important bearing on the nutrition of the mucosa of the ischemic mucosa *e g* during shock The low blood flow in such a situation may imply a still more reduced oxygen supply to the villi with the risk for hypoxic lesions as a consequence

Summary and conclusions

1 The wash out of intra-arterially injected Kr^{85} from denervated small intestinal segments (mainly jejunum) of the cat was recorded by means of a scintillation detector at various levels of vascular tone induced by graded infusions of isopropylnoradrenaline Total intestinal blood flow was simultaneously recorded by means of direct continuous *measurements* of the venous effluent At all levels of total intestinal blood flow the tracer elimination curve could be resolved into four components by successive subtractions of exponentials

2 The localization of the tissue compartments corresponding to the four components of the intestinal γ curve was established by the following four independent methods

a The elimination of the Kr^{85} β activity was registered by two G M tubes one located outside the intestine at the antimesenteric border (external G M tube) and one placed in the intestinal lumen (internal G M tube)

b Local injections of minute amounts of a Kr^{85} solution were performed into different parts of the intestinal preparation and the elimination of the tracer was recorded by a G M tube

c The wash out of antipyrine C^{14} was investigated by means of autoradiography

d The calculated relative weights of the two slowest compartments of the γ curve were compared with the measured relative weights of the muscularis and the mesentery

The different techniques described above gave consistent results as to the localization of the different components The most rapid component of the intestinal γ-curves predominantly reflected a countercurrent exchange of krypton between the ascending and descending limbs of the vascular loops in the mucosa at low and moderate blood flow levels (see below) During vasodilatation on the other hand when the efficiency of a countercurrent exchanger can be expected to diminish because of the high linear flow rate the most rapid component mainly mirrored the wash out of the tracer from a now particularly well perfused section situated in the submucosa and adjacent parts of the mucosa The second and third components mainly reflected the disappearance of Kr^{85} from the other sections of the mucosa and from the muscularis respectively The slowest component probably

mirrored an elimination of Kr^{85} from the perivascular fat of the mesentery and an absorption of the tracer from the intestinal lumen

3 The following patterns of blood flow distribution in the intestinal wall were made probable with the Kr^{85} wash out technique

a At resting levels of intestinal blood flow (30—35 ml/min × 100 g of tissue) most of the blood supply (60—80 per cent) was diverted to the mucosa to give an average blood supply to this section of 40—60 ml/min × 100 g Only 15—25 per cent of the intestinal blood flow was delivered to the muscularis corresponding to a blood supply of 10—15 ml/min × 100 g of this tissue 5—10 per cent of the intestinal blood flow seemed to be distributed to a small but well-perfused tissue section located in the submucosa and the immediate surroundings of the intestinal crypts where the average blood flow appeared to be as high as 400—600 ml/min × 100 g

b During intense vasodilatation (total intestinal blood flow 200—250 ml/min × 100 g) produced by isopropylnoradrenaline infusion the intestinal blood flow pattern became markedly altered As much as 50—60 per cent of the total flow was then distributed to the very well perfused tissue section in the submucosa and around the crypts which had now also greatly increased in size where blood flow levels as high as 800—1000 ml/min × 100 g were measured About 25—35 per cent of total intestinal blood flow was delivered to the mucosal vascular bed, giving here an average flow level of 150—200 ml/min × 100 g Slightly less than 10 per cent of the total flow was diverted to the muscularis corresponding to 35—40 ml/min × 100 g of this tissue

4 The following observations made in the present study constitute evidence for the existence of a countercurrent exchange of material between the ascending and descending vascular limbs of the mucosal vascular loops mainly located in the villi

a The blood flow value as calculated from the components of the Kr^{8} wash-out curve was compared with that of the directly and simultaneously recorded total intestinal blood flow It was found at low and moderate flow levels that only parts of the first component of the γ curve had to reflect true blood flow to account for the recorded total intestinal blood flow Extravascular events such as a countercurrent exchange, may thus be reflected in the first component of the γ curve

b The localization of the fastest rates of wash out *i e* the first component of the Kr^{85} γ curve seemed to move from the intestinal tissue around the crypts towards the tips of the villi as total intestinal blood flow was increased by infusion of isopropylnoradrenaline

c At resting levels of intestinal blood flow the venous appearance

time of intra-arterially injected red cells labelled with methemoglobin exceeded that of oxygen administered in an identical manner by 1—2 sec This observation is taken to indicate an extravascular shunting of oxygen between the limbs of the mucosal vascular loops

d Intravascularly administered lipid soluble substances (antipyrine 4 iodoantipyrine) were to a considerable extent excluded from the villi at low levels of intestinal blood flow A similar phenomenon although less pronounced was observed after intravascular administration of lipid insoluble solutes (rubidium, urea) These findings can be explained by a countercurrent exchange of the substances creating a relative hindrance for the material to reach the apical parts of the villi

e A comparatively slow wash out of antipyrine krypton and urea from the villi was demonstrated at low and moderate levels of total intestinal blood flow According to the countercurrent hypothesis these observations are explained by the exchanger acting as a barrier for the elimination of solutes from the tissue supplied by the hairpin vascular loop

f The internal G M tube registered a slow continuous increase of activity at resting intestinal blood flow in some experiments lasting up to 10 min after a slug injection of Kr^{85} This initially puzzling finding is explained in terms of the countercurrent hypothesis by a redistribution of Kr^{85} within the villi Immediately after the tracer injection the concentration of Kr^{85} will be greatest at the bases of the villi while the hairpin loop arrangement tends to establish a reverse concentration gradient

5 No appreciable countercurrent exchange of lipid soluble material (antipyrine krypton) appeared to take place when total intestinal blood flow exceeded 150 ml/min × 100 g The countercurrent exchange diffusion of urea (lipid insoluble) already seemed to become insignificant when intestinal blood flow was approximately 100 ml/min × 100 g These observations are in agreement with the fact that countercurrent exchange across a hairpin loop will gradually become reduced the more the linear velocity increases along the loop

6 Some functional implications of the present findings particularly with respect to intestinal absorption have been discussed

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 304

RESPIRATORY REGULATION DURING POSTNATAL DEVELOPMENT IN CATS AND RABBITS AND SOME OF ITS MORPHOLOGICAL SUBSTRATE

BY

GÖSTA H SCHWIELER

STOCKHOLM 1968

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 304

From the Department of Anatomy Karolinska Institutet Stockholm 60 Sweden

RESPIRATORY REGULATION DURING POSTNATAL DEVELOPMENT IN CATS AND RABBITS AND SOME OF ITS MORPHOLOGICAL SUBSTRATE

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ERRATA

Page 36 line 23 and in text of Fig 7 For "2000" read "1300"
Page 36 scale under Fig 7 For "5 μ" read "10 μ"

The expression "or NH_4Cl" should be deleted

on page 87	line 11
90	15
91	11
100	29
111	29

Page 105 line 36 For "Fig 37" read "Fig 36"

STOCKHOLM 1968

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Preface

The present work has been mainly carried out at the Department of Anatomy Karolinska Institutet and I wish to extend my sincere thanks to Professor Ture Petren Head of the Department for his personal interest and willingness to supply me with the facilities of his Institute

I got my first years of scientific training at the Department of Human Anatomy University of Uppsala and I take the opportunity to thank Professor Bror Rexed then Head of the Department for the time spent in the stimulating atmosphere of his laboratory

To Associate Professor Sten Skoglund, who introduced me into experimental anatomy and physiology and originally suggested the subject of this study I wish to express my very deep gratitude His stimulating interest generous personal support and able criticism have meant everything for carrying through this work

My thanks are also due to my colleagues in the Departments for their assistance and for many valuable informal discussions Finally I would like to express my heartfelt thanks to the members of the technical staff for much skilful help and especially to Miss Maj Beghman for drawing all the illustrations Miss Anette Wijkman for typing the manuscript and Mr Rudolf Rudefjell for manufacturing several pieces of the experimental equipment

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Stockholm November 1967

Gosta H Schwieler

General introduction

During recent years considerable advances have been made in the understanding of the regulation of respiration in the adult Relatively little attention has been paid however to the problems of respiratory control during postnatal development Interest has been focused mainly on the problem of how respiration is initiated since it has often been suggested that once respiration in the newly born has started it is controlled in the same way as in the adult (see James and Adamsons 1964 a b Cross 1965 Dawes 1965 Purves 1967) Very little experimental evidence has been presented however in support of this view Coombs and Pike (1930) actually showed that newborn kittens and puppies in contrast to adult animals are unable to survive section of the phrenic or the vagus nerves further respiration in the newly born seems to be more sensitive to hypoxia (see Cross 1964) and probably also to hypercapnia (Stahlman 1961 Avery Chernick Dutton and Permutt 1963)

Skoglund (1960 a—e) has shown that in the kitten a considerable postnatal development of the reflex activities takes place Thus there is no tonic stretch reflex in the distal hindlimb muscles of the newly born but it appears later with the development of the functional properties of the afferents of the muscle spindles and the appearance of their gamma" innervation The suggestion of Skoglund that there is a correlation between the development of reflex activity and the development of the nerve fibres was supported by Wilson (1962) Recently it has been shown that there is a correlation between morphological and functional changes in the developing nerve fibres (Skoglund and Romero 1965 Berthold and Skoglund 1965 1967) In view of these findings it appeared worth while to reconsider some of the mechanisms of respiratory regulation during postnatal development

Since very little experimental work has been done previously on the postnatal development of respiration it was necessary to approach to the problem on a relatively broad front Thus the aim of the present investigation has been to study which of the respiratory control systems known to be of importance in the adult animal are already functional at birth or if as yet immature how they develop postnatally As a starting point for a further experimental analysis the effect of vagotomy has been reinvestigated The experimental animals were mainly cats but some rabbits were also used

In order to clarify the background of the present investigation a short survey of the literature will be given Some of the aspects of respiratory control in the

adult will thus be reviewed and discussed in relation to earlier experimental results in the newly born Some recent advances with regard to neuronal maturation will also be discussed since a similar approach has been used here for studying the developing regulation of respiration The following main questions are treated

1) Which of the respiratory control systems are functioning at birth and how can the deleterious effect of vagotomy in the newly born be explained?

2) Does the correlation between functional and morphological maturation which has been found with regard to different somatic afferent systems also hold true for afferents of direct importance in respiratory control?

3) Can possible differences in sensitivity of the respiratory system to hypoxia or hypercapnia in the newly born be attributed to the receptor systems which are known to respond to such changes in the adult or to more generalized effects on the developing neurons?

The aim has been to obtain answers to these main questions from the results of a series of defined experimental questions that are presented in the respective chapters of this monograph

Literature survey

In adult mammals there are two main respiratory regulation systems One system serves to control the respiratory pattern i e the frequency and mechanical force of breathing in relation to changes in mechanical properties of the lungs airway resistance and posture of the body The most important receptors known to be engaged in this control are the pulmonary tension receptors (see Widdicombe 1964) and the intercostal muscle spindles (see Euler 1966) The other respiratory regulation system serves to adapt ventilation to chemical changes in the blood such as pO_2 pCO_2 and pH Whereas a change in the oxygen tension of the blood changes ventilation via the peripheral chemoreceptors located in the carotid aortic and possibly pulmonary glomerula (see Comroe 1964) changes in the blood pCO_2 and pH act primarily via central chemoreceptors located in the medulla oblongate (see Kellogg 1964)

1 Regulation through mechanoreceptors

a) Adult mammals

The probably best known modulator reflex whereby the respiratory pattern is controlled is that which tends to terminate the inspiratory effort and shorten the respiratory cycle and which is mediated from the tension receptors within the lungs via afferents in the vagus nerves the so called Hering Breuer inflation reflex" (Breuer 1868 Hering 1868 Adrian 1933) The physiological importance of this reflex has been much discussed (see also Wyss 1964) Originally a Selbststeuerung" hypothesis was developed by Hering and Breuer (1868) Although this hypothesis has suffered some neglect due to the discoveries of other reflexes of equal importance for the control of respiration and with the increasing knowledge of the central respiratory regulation system (see Wang and Ngai 1964 and Widdicombe 1964) it is clear that the reflex nevertheless influences respiration in important respects Evidence has been presented that this reflex keeps the respiratory frequency at an optimal value at which the work of the respiratory muscles is minimal (see Widdicombe 1964)

In a series of recent works Euler and co workers (see Euler 1966) have shown that also the thoracic dorsal root afferents partake in the regulation of the respiratory pattern Thus during each inspiration the intercostal muscle spindles appear to exert a facilitation on the thoracic motoneurons which decreases with increasing thoracic volume This receptor system appears to sense whether the central de

mand" with regard to the tidal volume is fulfilled (see Campbell and Howell 1962) and thus if the shortening of the intercostal muscles is adequate in relation to the changes in airway resistance body position etc Each contraction seems to be initiated by stimulation of the "gamma or fusimotor neurons which cause the intrafusal fibres to contract whereby the spindles are stretched and the resultant sensory discharge drives the alfa neurons and extrafusal muscle fibres (see Corda Eklund and Euler 1965) Whereas muscle spindles seem to be rather sparse in the diaphragm (Corda Euler and Lennerstrand 1965) the spindle afferents of the lowermost thoracic segments also affect the phrenic motoneurons in the same way as the intercostal ones (Decima Euler and Thoden 1967)

b) Newborn mammals

It has been reported that the "Hering Breuer inflation reflex is present at birth in rabbits lambs monkeys and man (Dawes and Mott 1959 Hughes Parker and Williams 1967 Dawes Jacobsson Mott and Shelly 1960 Cross Klaus Tooley and Weisser 1960) The observation of Coombs and Pike (1930) that section of the vagus nerves affects respiration more in the newly born than in the adult indicates that the Hering Breuer inflation reflex is of greater importance for the respiratory control in the early stages of postnatal development However no report has been found with regard to the afferent activity from the pulmonary tension receptors during postnatal development

Although there seems to be no detailed information about the role of the thoracic dorsal roots in the newly born Coombs and Pike (1930) stated that the "costal respiratory movements are relatively slighter in kittens" and that these animals in contrast to adults do not survive section of the phrenic nerves i e they are unable to compensate for diaphragmatic failure with thoracic respiration These observations appear to be of significance in the early stages of development since they point to a lack of the respiratory control which in the adult animal is exerted by the intercostal muscle spindles

2 Regulation through changes in blood oxygen tension

a) Adult mammals

It is well known that a decrease in arterial pO_2 caused for example by a decrease in the relative partial pressure of oxygen in the inspired air to 10 % (air=21 % O_2) gives rise to a sustained hyperventilation in the adult by a stimulation of the peripheral chemoreceptors (see Bjurstedt 1946) If these receptors are denervated a moderate degree of hypoxia does not affect ventilation at all whereas a more pronounced hypoxia leads to a depression of ventilation (Gemill and Reeves 1933)

If on the other hand the oxygen concentration of the respired gas is increased above 21 % (air) respiration decreases indicating that the peripheral chemore ceptors in the adult continually

Thus by administering 100 % O_2 a "physiological denervation" of the peripheral chemoreceptors is achieved (Dripps and Comroe 1947)

b) Newborn mammals

The evidence regarding the function of the peripheral chemoreceptors at birth in man and different mammalian species is conflicting Boyd (1937) stated that the carotid and aortic chemoreceptors are morphologically mature long before birth in man Only two reports of direct recordings from the afferent nerves of these receptors seem to exist Thus Cross and Malcolm (1952) found an increase in impulse activity in the sinus nerve in full term fetal and newborn lambs and kittens and rabbits some 2—3 weeks of age on exposure of the mother or the young animal to low partial pressures of oxygen Very recently Biscoe and Purves (1967) have confirmed the finding that hypoxia increases the discharge from the peripheral chemoreceptors in newborn lambs The recordings however were in both cases made from large strands of the sinus nerve No recordings from single units seem to have been made

Indirect studies are much more numerous The respiratory response to injections of different substances and to changes in the oxygen content of the respired air have been studied Whereas injection of cyanide into full term fetal lambs seems to be ineffective in producing a respiratory response (Barcroft and Karvonen 1948) it does elicit such a response in lambs shortly after birth or if some other sensory stimulus is set up in the fetus simultaneously with the injection of the cyanide (Reynholds and Mackie 1961) In the newborn lamb the threshold for the response decreases if the oxygen supply is increased (Reynholds and Mackie 1961) Newborn rabbits also respond with a transient respiratory augmentation after an intravenous injection of sodium cyanide lobeline or nicotine (Dawes and Mott 1959) This response was found to be present only if the sinus and vagus nerves were intact and consequently it was concluded that the response is reflexly mediated from the peripheral chemoreceptors

It has been reported that newborn infants and lambs respond to pure oxygen in the same way as the adult i e with a ventilatory diminution (Cross and Warner 1951 Cross and Oppe 1952 Brady Cotton and Tooley 1964 Purves 1966 a) which indicates that the receptors are active during airbreathing Other workers however have not found such an initial diminution but only a progressive increase in ventilation (Miller 1954 Girard Lacaisse and Dejours 1960) These latter authors thus maintain that either the receptors are not functioning at birth or are in some way unable to influence the respiratory center

Finally the effect of decreasing the oxygen concentration of the respired air has been studied While some authors have found that respiration is only depressed (Miller and Behrle 1954 Miller and Smull 1955) most workers agree that in the newborn there is at least an initial increase in respiration in response to hypoxia (Cross and Warner 1951 Cross and Oppe 1952 Cross Hooper and Lord 1954

Dawes and Mott 1959 Brady and Ceruti 1966) although the hyperventilation is only maintained if the environmental temperature is neutral (i e the temperature at which the oxygen consumption is minimal Adamsons 1959 Dawes and Mott 1959) Otherwise respiration is subsequently depressed It must be pointed out however that from the diagrams and records presented by Adamsons (1959) and Dawes and Mott (1959) it appears that in rabbits respiration tends to decrease progressively after the initial increase also at neutral temperature although the ventilation is better maintained than in a cold environment

In conclusion it may be stated that there is evidence for the view that the peripheral chemoreceptors are functionally active in the newborn However re cordings from single receptors do not appear to have been made and it has *never been finally settled whether the ventilatory depression on hypoxia is due* to a central depression or to cessation or decrease in the receptor discharge Further more some metabolic factor appears to be involved since the response depends partly on the temperature Adamsons (1959) pointed out that the CO_2 production might decrease whereby the central drive is diminished

3 Regulation through changes in blood pH and pCO_2

a) Adult mammals

It is a well established fact that blood acid base changes affect respiration In the adult it is known to be stimulated by a decrease of pH of the blood Thus both by administering CO_2 in the respired air and by injecting acid solutions into the blood ventilation is increased (see Kellogg 1964) Conversely a raised blood pH depresses respiration as can be demonstrated by injecting a basic solution into the blood (see Hesser 1949 and Singer Deering and Clark 1956) Under physio logical conditions the respiratory system displays the most rapid response (see Cort 1965) of those systems involved in maintaining a constant acid base balance of the blood and extracellular fluids An accumulation of acid metabolities increases ventilation whereby the CO_2 consequently formed is expelled and the blood thereby indirectly buffered ($H^+ + HCO_3^- \rightleftarrows H_2CO_3 \rightleftarrows H_2O + CO_2$) Conversely when basic metabolities accumulate there is a decrease in ventilation

It has not not been finally settled how this control of the ventilation is achieved Since Haldane and Priestly (1905) published their studies regarding the CO_2 induced changes in respiration two main problems have been discussed viz the location of the responsible chemoreceptors and the adequate stimulus of these receptors

It is now generally held that at least in the adult both the peripheral chemore ceptors located in the carotid and aortic glomerula and the central receptors assumed to be in the medulla oblongata mediate in the regulation of ventilation through blood acid base shifts (see Kellog 1964) As regards the relative contribu

tions of these two sets of receptors under normal conditions however there is considerable divergence of opinion (see e g Katsaros 1965 Mitchell Carman Severinghaus Richardson Singer and Shnider 1965 Pappenheimer Fencl Heisey and Held 1965 Davies and Yamamoto 1966)

There is also controversy with regard to the precise site and character of the central chemoreceptors Although they appear to be located in the medulla oblongata the exact morphological structure for these receptors has not been found (see Loeschcke 1965) Indirect evidence suggests they may be located at or near the bottom of the fourth ventricle on the ventrolateral surface of the medulla and within the medullary neural tissue proper It has also been proposed that the neurons of the respiratory reflex and coordination centers in the medulla are chemosensory themselves (for further discussion and references see Kellogg 1964) Whereever they are located there appears to be ample evidence that the acid base balance not only in the blood but also in the medullary extra cellular or cerebrospinal fluid (C S F) affects these receptors thereby changing ventilation (see Brooks Kao and Lloyd 1965) It must also be pointed out that the excitability of nervous and excitable tissue in general is affected by acid base changes in their environment (see Wyke 1963) But whereas most neurons appear to be depressed by acidosis and excited by alkalosis evidence has accumulated that the opposite holds true for the neurons of the "reticular activating system" (see Wyke 1963) which should thus instead be activated by an increase in pCO_2 and/or decrease in the pH of their environment This might well be the explanation for the arousal reaction seen as a desynchronisation of the EEG on exposure of animals to a moderate increase in CO_2 of the respired air and also for the concomitant respiratory augmentation obtained (for further references and discussion see Wyke 1963)

With regard to the adequate stimulus finally it has been shown that both a change in blood pH with the pCO_2 constant and a change in pCO_2 with a constant pH both affect respiration whether the peripheral chemoreceptors are intact (Lambertsen Semple Smyth and Gelfand 1961) or denervated (Katsaros 1965) Since however the chemosensory receptors appear to be at least partly located extravascularly (see above) it cannot be excluded that e g a change only in blood pCO_2 causes a change both in pCO_2 *and* pH in the immediate vicinity of such receptors (see Lambertsen Semple Smyth and Gelfand 1961) However from experiments where unanesthetized goats were perfused through the ventricular system with different "artificial" cerebrospinal fluids and the blood values simultaneously controlled Pappenheimer and co workers (see Pappenheimer Fencl Heisey and Held 1965 Fencl Miller and Pappenheimer 1966) concluded that the resting ventilation is a single function of the hydrogen ion concentration in the cerebral interstitial fluid Since this conclusion is based on indirect evidence further experimental support is required before it can be accepted

b) Newborn mammals

With regard to the newborn there is very little experimental evidence to show whether the central chemoreceptor system is already in function or if it undergoes some postnatal development The respiratory response to inhaled CO_2 has been studied in newborn infants and lambs (Cross Hooper and Oppe 1953 Stahlman 1961 Avery Chernick, Dutton and Permutt 1963 Purves 1966 c) In only one case (Purves 1966 c) were the blood acid base parameters measured simultaneously Although the general finding has been that respiration increases in response to an increase of pCO_2 in the respired air some authors (Stahlman 1961 Avery Chernick Dutton and Permutt 1963) report that there appears to be a stronger tendency to accumulate CO_2 in the newborn and that respiration as a consequence is depressed It has also been reported that acidotic newborn infants do not always hyperventilate as do adults (Bland 1956) Thus although in the newborn CO_2 appears to stimulate ventilation it also appears that respiratory depression may more easily occur but no further analysis of this problem seems to have been made Since in no experiment the peripheral chemoreceptors were completely denervated it cannot be excluded that the stimulation of ventilation is only mediated by these receptors which also at least in the newborn lamb have been shown to increase their discharge frequency in response to CO_2 (Biscoe and Purves 1967) Thus there appears to be a dearth of information about the function of the central chemoreceptors

The normal acid base balance of the blood has also been studied in man and lambs during postnatal development It has been a common finding that in the newborn the blood acid base pattern after the first 24 hours of extrauterine life is that of a compensated respiratory alkalosis Thus the pH is normal whereas both the arterial pCO_2 and $[HCO_3^-]$ are reduced (Albert and Winters 1966 Purves 1966 c) From this it has been proposed that some kind of "excess stimulus of respiration which causes CO_2 to be expelled exists at this stage It must firstly however be settled whether there are any qualitative differences between the newborn and the adult with regard to the effect on respiration of changes in blood acid base values Not until this is known does it appear worthwhile to discuss quantitative comparisons Moreover the possibility exists that the CO_2 production in the newborn is relatively less and that this is the reason for the alkalosis found

One observation which might be of importance with regard to the control of ventilation through blood acid base shifts is the finding of a lower blood carbonic anhydrase activity in the newborn infant (Berfenstam 1952) Berfenstam found that the enzyme activity in the blood of premature infants was about 5—10 % that of adults Although Roughton (1945) calculated that only 1 % of the carbonic anhydrase activity of the adult was necessary for adequate CO_2 elimination through the lungs this might not be so in the newborn since important differences exist with regard to histological structure vascular supply and state of

function of the lungs (atelectasis respiratory surface etc) as pointed out by Berfenstam (1952)

Summarizing briefly it may be stated that indirect evidence favours the view that the pulmonary tension receptors and the peripheral chemoreceptors are functioning already in the newly born but that nothing appears to be known about the importance of the thoracic afferents at this stage Further it appears that hypoxia as well as possibly hypercapnia tends to depress ventilation more easily in the newly born than in the adult and finally that nothing appears to be known about the function of the central chemoreceptors in the newborn stage

4 Postnatal development of some neural mechanisms

In recent years evidence has been published which elucidates important differences between the newborn and adult cat with regard to neural function Some of these observation may have direct bearing on the problems of respiratory control during postnatal development Thus different somatic receptors have been found unable to fire continually in response to a maintained stimulus in the newborn kitten (Skoglund 1960 c Ekholm 1967) Indirect evidence favours the view that the aquisition of this feature is dependant on the maturation of the afferent nerve fibres Thus when the fibres have reached a diameter of some 3 5—4 microns i e a conduction velocity of some 20 m/sec the adult refractory period is reached as well as the ability to transmit impulses continually (Skoglund 1960 b c Ekholm 1967) Moreover at this stage the node paranode region of the nerve fibre (Berthold and Skoglund 1968) appears to be qualitively mature

Further in newborn kittens distal hindlimb muscle spindles have been shown to lack "gamma control (Skoglund 1960 c) This gradually develops and is established some 17—20 days postnatally when a tonic stretch reflex can be demonstrated in these muscles A "gamma" elevation can be seen in the neurogram some 10 days after birth (Skoglund 1960 b) and soon also a peak of "gamma fibres appears in the fibre calibre spectrum of lumbar ventral roots (Skoglund and Romero 1965) Moreover Zelena (see Zelena and Hnik 1963) has shown that the "gamma" fibres do not reach the muscle spindles until some time after birth in rats

During postnatal development there appears to be a great increase in the number of mitochondria in the central nervous system (Samson Balfour and Jacobs 1960 Skoglund 1967) as well as in peripheral nerves (Berthold and Skoglund 1967) This may be of functional significance as exemplified by the finding that when the mature pattern of Schwann cell mitochondria in the peripheral nerves is reached the fibres are also functionally mature(Berthold and Skoglund 1967)

General metods

I *Anesthesia*

Cats and kittens As a rule pentobarbital sodium (Nembutal Abbott) in a 1 % or 2 % solution (1 or 2 g/100 ml Ringer s solution) was given intraperito neally (i p) It is well known that all barbiturates depress ventilation and de crease the sensitivity for example to CO_ (see Severinghaus and Larsson 1965) In the present work kittens below 2—3 weeks of age were found to be particu larly susceptible in this respect Thus at the weight dose usually given to adults (40 mg/kg b w =body weight) the sensitivity to CO_2 (see Chapter VIII) often disappeared completely as seen from the absence of a ventilatory increase in re sponse to administration of 3 % or 6 5 % CO_2 in the respired air (Fig 1) The reflex sensitivity to pain on the other hand was not so easily depressed by the barbiturate and the dose had to be chosen so that ventilation was minimally affected but yet kept the animal sufficiently anesthetized for experimentation Often a single i p dose of 30—35 mg/kg b w was suitable but occasionally also this depressed ventilation considerably with an ensuing respiratory acidosis and a later ventilatory depression (see Chapter VIII) To be able to lower the dose of barbiturate still further a combination of partial anemic decerebration and 20—25 mg/kg b w Nembutal was tried in some animals The common carotid arteries were firstly ligated under ether anesthesia after which the barbiturate was injected intraperitoneally Such animals constantly responded to CO_2

In the present investigation only kittens which irrespective of the dose of Nem butal responded with an increase in ventilation during a short period (1—2 min)

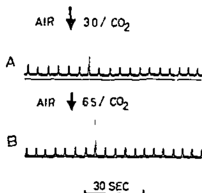


Fig 1 Respiratory record (intraoesophageal pres sure swings inspiration deflection upwards) from a 7 days old kitten anesthetized with 40 mg/kg Nembutal The administration of 3 % and 6 5 % CO_2 in the respired air (at the arrows) did not stimulate respiration

of breathing air containing 3 % CO_2 were used Occasionally a small second dose of Nembutal (5 mg/kg) was given intravenously (i v) during the course of an experiment to keep the animal quiet This was done not less than 30 minutes before the beginning of a test period As a rule no additional anesthetic was required even though the experiment might last several hours

To animals older than 4 weeks an i p dose of 40 mg/kg b w Nembutal was regularly given All these animals responded to 3 % CO_2 in the respired air Small (5—10 mg/kg) further doses were given at intervals of 1—2 hours which maintained the animals roughly at the same anesthetic level All such extra doses were given not less than 30 minutes before a test period The anesthetic level must be expected to vary somewhat during an experiment due to the continuous metabolic breakdown of the anesthetic Such variations were slight however and do not seem to be of any importance for the general interpretation of the results obtained Urethane and chloralose were also tried but were found to depress ventilation too strongly in the kittens below 2—3 weeks of age when anesthetic doses were given

Rabbits Rabbits were given urethane in a 50—100 % solution A single i p dose of 1 2—1 8 g/kg b w was constantly found adequate to induce sufficiently deep anesthesia At this dose animals of all ages responded with an increase in ventilation to 3 % CO_2 in the respired air Thus cats and rabbits seem to react very differently to urethane No further doses of urethane had to be given during the experiment

II *Decerebration*

To obviate the influence of any anesthetic agent decerebrated cats and rabbits were also used Under ether anesthesia given through a tracheal cannula the animals were decerebrated by section or suction above the superior colliculi The common carotid arteries were always tied previously Half an hour or more was then allowed to elapse before the experiment was continued Although ventilation in cats and kittens was most often adequate to keep the arterial pH and pCO_2 within normal adult values (pH about 7 35 pCO_2 about 35 mm Hg see Dittmer and Grebe 1958) it was often irregular and therefore the decerebrate preparation was not very suitable for these studies However there were sufficient periods when ventilation was stable to confirm that in general the results obtained on anesthetized animals were the same in decerebrate and hence unanesthetized animal In the reflex investigations in Chapter VII decerebrate animals were always used

III *Recording of respiration*

Respiration was recorded by following the intraoesophageal pressure variations which reflect rather well the intrapleural pressure changes (see Mead and Milic Emili 1964) An open ended water filled polyethylene cannula connected to a

strain gauge manometer (I Ljungstrom Sweden) was introduced into the oesophagus The strain gauge transducer was connected to a Vibration analyzer (type TL 21 F T Ljungstrom) and the pressure changes were displayd on an oscilloscope screen and photographed on moving bromide paper The transducer was calibrated and found to be linear within the actual range of oesephageal pressure changes (0—20 cm H_2O) The polyethylene cannula coated with Xyla cain Gel (Astra) was inserted to a level just below the point where the heart beats did not interfere with the ventilatory pressure changes (i e about 5 cm in the newly born 10 cm at two weeks 15 cm at six weeks and 20—30 cm in the adult animals etc measured from the lips) In Chapters V and VII only a qualitative indication of the respiratory cycle was necessary so for these chapters no more comment on the method seems necessary In Chapters IV VI and VIII on the other hand an indication of possible changes in ventilation caused by different denervation procedures by changes in the composition of the respired air etc was desired In the literature the minute volume (tidal air times fre quency) is as a rule used as a measure of pulmonary ventilation (see e g Kellog 1964) Since the volume pressure curve for volume changes within physiological limits is generally a straight line (Buytendijk 1949 Radford 1964) the amplitude of the pressure swings should reflect rather well the volume in spired Thus the product of pressure amplitude and frequency per minute has been used here as an index of ventilation (Ve) In the figures the values of ventilation thus calculated are given as percentage deviations from the value ob tained during the initial steady state period the latter value being denoted as zero Since in this work (Chapters IV VI and VIII) only changes in ventilation in response to different stimuli were studied no absolute values of the minute volumes were required Thus the fact that a small animal inhales a smaller volume than an adult one in response to the same intrathoracic pressure change does not affect the interpretation of the present results nor does the possibility that the pulmonary compliance and thus the slope of the pressure volume curve differs between ani mals of different ages (see Agostoni and Mead 1964) The effect of a possible change of the pulmonary compliance during an experiment will be discussed whenever pertinent

The reason for using the above method and not a spirometer for recording respiration was not only because it was much easier to handle in combination with the rest of the relatively complicated recording systems but also because of the pronounced technical difficulties in constructing a spirometer for the small new born animals without too large a dead space or resistance to breathing The main reason though was that the problems studied here do not require any exact re cording of the minute volume or its changes

IV *Recording of blood pressure*

The femoral artery was cannulated with a polyethylene tube filled with Ringer s

CHAPTER III

Some basic observations

The following data are presented in order to give an idea of the normal values and their ranges with regard to breathing frequency for comparison with the present experimental findings In addition some data on the blood acid base values in anesthetized and decerebrated animals of different ages are given

I *Breathing frequency and its relation to anesthesia in cats of different ages*

In unanesthetized animals the number of breaths per minute was with the aid of a stop watch counted by direct inspection The breathing frequencies in anesthetized and decerebrate animals were counted from the records of the intra oesophageal pressure oscillations (see page 18) All animals breathed room air

At all ages the respiration was highly irregular in conscious freely moving animals with frequencies varying from some 20/min to more than 100/min In resting animals the respiratory frequency was more constant although still varying considerably In Fig 3 the means and ranges of the frequency counted as described above on 20 different occasions in 5 resting cats of different ages are illustrated It can be seen that the frequency is highest in the youngest animals and decreases with age In the 6 weeks old animal the adult value is reached

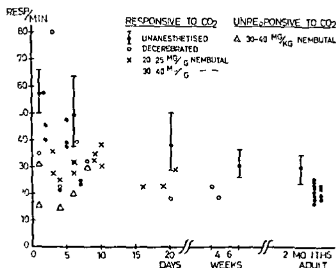


Fig 3 Breathing frequencies in unanesthetized anesthetized and decerebrate cats of different ages breathing room air (see text)

Fig 3 also gives for comparison the breathing frequency in anesthetized and decerebrate animals of different ages generally respiration frequencies were higher at lower ages But in comparison with the older ones most of the young cats seemed to be more depressed by the anesthetic and/or surgical procedure The variation was of about the same order in decerebrate as in anesthetized animals in spite of the fact that the latter regularly showed better arterial pH and pCO_2 values (see below) Most of these animals responded on a short exposure to 3 % CO_2 in the respired air by an increase in ventilation a few that did not respond in this way are indicated separately

In conclusion the breathing frequency seems to decrease postnatally from some

Table I Arterial pH and pCO values and the breathing frequencies in some anesthetized and decerebrated cats and kittens of various ages

An sthet zed animals			
Age in days	Arterial pH	Arterial pCO	Bre ths per min
1	7 33	50	44
2	7 30	54	26
2	7 30	50	40
[illegible]	7 30	54	34
2	7 39	45	[illegible]
5	7 34	47	48
8	7 36	49	28
10	7 35	47	31
11	7 33	50	38
20	7 32	43	34
60	[illegible]	46	2
90	7 45	34	18
150	7 4	43	16
150	7 38	[illegible]	24
adult	7 35	35	16
Decerebrated animals			
1	7 40	41	35
	7 48	30	42
4	7 47	31	2
[illegible]	7 38	39	38
20	7 39	36	20
31	[illegible]	34	18

50—70/min shortly after birth in unanesthetized resting animals to some 25—30/min in adults this value being reached in the 6 weeks old animal The anesthesia and/or surgical procedure seems to cause a relatively more pronounced decrease in breathing frequency in the youngest animals However from the breathing frequency alone no conclusion can be drawn about degree of depression of the respiratory center" or its sensitivity to CO_2 since some of the animals which did respond to CO_2 showed lower frequencies than some of those which did not

II *The arterial pH and pCO_2 values in anesthetized and decerebrate cats of different ages*

Table I shows the arterial pH and pCO_2 values from a number of decerebrate and anesthetized animals of different ages The breathing frequency is also included The values were recorded when the animals breathed air and before any experimental procedure had been begun All these animals responded by an increase in ventilation during a short exposure to 3 % CO_2 in air From the Table it is clear that the anesthetized animals below 3 weeks of age showed generally more acidotic values than the decerebrated or the older anesthetized animals The values also illustrate the lack of correlation between the blood acid base values and the respiratory frequency

As pointed out above the animals below 3 weeks of age were generally given a relatively smaller dose of Nembutal than the older ones In spite of this they became more acidotic which indicates that their respiration was more sensitive to the barbiturate It is also possible that they are normally more acidotic This seems unlikely however since other newborn animals and infants after the first 24 hours of extrauterine life show a compensated respiratory alkalosis (see Purves 1966 c)

The effect of sectioning the vagus and sinus nerves on respiration in cats and rabbits during postnatal development

Introduction

During a study of the neuromuscular mechanisms of respiration in cats and dogs Coombs and Pike (1930) noticed that vagotomy seemed to affect respiration more in the newborn and that death due to respiratory failure supervened within one or a few hours They reported that sometimes in particular the diaphragmatic activity recorded as the movements of the abdomen was reduced

There is an extensive literature on the pulmonary effects of vagotomy and on the subsequent survival time (see Tigyi and Lissak 1960 Shanklin and Sotelo Avila 1967) Thus mice rats rabbits guinea pigs cats and dogs of all ages develop a fatal pulmonary infiltration (Vaguspneumonie" Tigyi and Lissak 1960) and edema after cervical vagotomy In adult cats and dogs death occurs on the 3rd to 4th day in the other animals within the first 24 hours (Tigyi and Lissak 1960) The pulmonary changes after vagotomy have also been studied in newborn animals in which vagal section appears to result in the production of hyaline membranes (see Shanklin and Berman 1964) The survival time after vagotomy was correlated with the degree of the pulmonary changes (Shanklin and Sotelo Avila 1967) In view of the finding of Coombs and Pike (1930) that the respiratory activity is more severely affected by vagotomy in newborn animals the reason for the death might not only be sought within the lungs None of the authors studying lung changes and survival time in newborn animals after cervical vagotomy seem to have made systematical observations with regard to the changes in respiration (Shanklin and Sotelo Avila 1967)

The vagal nerves contain afferents from two different kinds of receptor systems known to be of direct importance for respiratory control the pulmonary mechano receptors and the vascular chemoreceptors (see Widdicombe 1964 and Comroe 19(4) There is some evidence that both these receptor systems are already

functioning at birth in rabbits lambs and man (Cross and Oppe 1952 Dawes and Mott 1959 Cross 1964 Purves 1966 a—c Huges Parker and Williams 1967) If interruption of their afferents cuts down respiration as reported by Coombs and Pike (1930) this indicates that respiratory control systems other than those conveyed in the vagi are either not functioning in the newborn or are unable to affect respiration when lacking the support of the vagal afferent input

Since the finding of Coombs and Pike (1930) does appear not to have been confirmed but rather denied (see Dawes 1967) it was considered of interest to reinvestigate the problem since it might throw some light on the most important differences between respiratory control in the newborn and the adult animal Decerebrate and anesthetized cats and rabbits of different ages were used In some cases the arterial pCO and pH and the blood pressure were recorded in addition to the respiration The effect of vagotomy on the respiratory pattern the ventilation and the arterial pH and pCO was first studied To test whether the effect of vagal section might in part be explained by the disappearance of facilitation from the aortic chemoreceptors the effect on respiration of denervation of the carotid chemoreceptors following vagotomy was also studied since the aortic and carotid chemoreceptors appear to influence ventilation in the same way To obviate as far as possible interference from possible changes within the lungs such as edema etc (see above) the changes taking place during the first hour after the vagotomy were primarily studied

The following questions were raised

1) Are there any differences between the newborn and the adult with regard to the change in respiratory pattern after vagotomy?

2) Is ventilation adequate after vagotomy at all ages i e are arterial pCO_2 and pH maintained at a constant level?

3) What is the ventilatory reaction to denervation of the carotid chemoreceptors following vagotomy at different postnatal ages?

Material and Methods

Sixty experiments were made on 20 kittens and 20 rabbits 1—21 days old and 10 cats and 10 rabbits between 4 weeks and 1 year old The concomitant changes in arterial pCO and pH were studied in 6 of the kittens below 2 weeks of age and in 3 of the cats 1—5 months of age

A cannula was first inserted into the trachea The cervical vagosympathetic trunks and the rami sinus carotici were then exposed bilaterally The ramus was most easily found where it joins the glossopharyngeal nerve where the latter traverses the tympanic bulla

Shortly before the vagi or the sinus nerves were cut they were coated with

Xylocain Gel (Astra) to prevent the generation of injury potentials The sym pathetic trunks we e always sectioned together with the vagi since connections be tween them are frequently found (cf Schwieler 1966)

Results

I *The effect of sectioning the vagus nerves on*

a) The respiratory pattern

When the cervical vago sympathetic trunks were severed respiration initially slowed down and the inspiratory phases were markedly prolonged both in an esthetized and decerebrate animals of all the ages studied from the newborn (ee Fig 4) With regard to the adult this change in breathing pattern after vagotomy is well known (see Wyss 1964) and it is commonly supposed to be due primarily to the disappearance of inhibition of inspiration from slowly adapting pulmonary tension receptors (Hering 1868 Breuer 1868 Head 1889 Adrian 1933) Since initially the same principal change in breathing pattern took place in newborn animals (Figs 4 B C) it might be concluded that this reflex is present already at birth in cats as well as in rabbits However as a rule the decrease in frequency seemed to be more pronounced in the youngest animals (Fig 4 B C) and whereas ventilation remained the same after as before the vagotomy in the animals above 4 weeks it appeared to decrease considerably in the animals below 2—3 weeks of age (see below) Moreover in these animals respiration sometimes became periodic or gasp like

The subsequent changes in the respiratory pattern caused by the vagotomy varied in the animals below 3 weeks of age and was partly dependant on whether they ware anesthetized or decerebrated The respiratory amplitude increased more in decerebrate than in fully anesthetized animals (Fig 4) Moreover in the decere brate and very lightly anesthetized (20—25 mg/kg b w Nembutal) ones respira tion sometimes became periodic after vagotomy with periods of extremely high amplitudes alternating with progressively longer periods of low amplitudes or later complete apnoe (Fig 4 C) This pattern sometimes continued for more than an hour but always ended with complete respiratory failure Occasionally this periodic pattern changed gradually into a gasping of low frequency as shown in Fig 5 D In animals anesthetized with 30—35 mg/kg b w Nembutal the breathing continued in a more regular fashion (Fig 4 B) and was generally maintained for a longer period of time

As pointed out above the ventilation appeared to decrease after vagotomy in the animals below 3 weeks of age Since however respiration sometimes became irregular this was somewhat hard to evaluate and it was decided to record also the arterial pCO and pH continuously to get a better measure of the adequacy (Severinghaus 1965) of the ventilation To ascertain also whether any gross

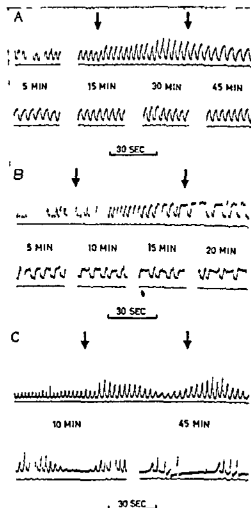


Fig 4 Respiratory records (intraoesophageal pressure swings inspiration deflection upwards) showing the effect of vagotomy At the first arrows the vagi were coated with Xylocain Gel at the second arrows they were cut (A) Kitten 4 weeks old decerebrate (B) Kitten 1 day old anesthetized with 30 mg/kg Nembutal (C) Kitten 7 days old decerebrate

changes in the circulation took place the blood pressure was also recorded

b) The ventilation and arterial pCO_2 and pH

Two typical experiments from a 4 months old cat and a 6 days old kitten are shown in Figs 5 A—D The older animal was anesthetized with 40 mg/kg b w Nembutal whereas in the kitten the carotid arteries had been ligated under ether and only 20 mg/kg b w Nembutal administred

In the 4 months old cat the vagotomy caused the typical decrease in breathing frequency and increase in amplitude (Fig 5 B) but only a transient slight decrease in ventilation after which the original value was again attained (Fig 5 A) The arterial pH and pCO_2 values remained constant during the experiment (Fig 5 A) which means that in spite of the change in respiratory pattern the ventilation was with regard to these parameters still adequate The blood pressure also

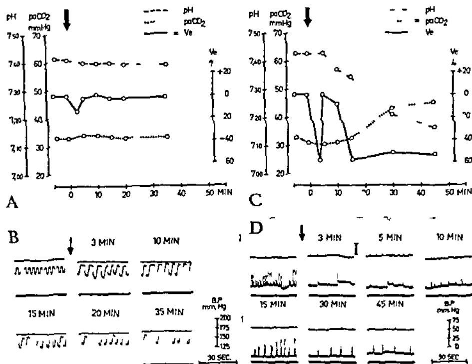


Fig 5 The effect of vagotomy (at the arrows) in a 4 months old cat (A B) (anesthetized with 40 mg/kg Nembutal) and a 6 days old kitten (C D) (anesthetized with 20 mg/kg Nembutal carotid arteries ligated) The diagrams (A C) show the arterial pH and pCO and ventilation The records (B D) show respiration (as in Fig 4) and the arterial blood pressure before and at different times after the nerve section Minutes indicate time after the vagotomy corresponding to the time scale on the diagrams

remained constant (Fig 5 B)

In the newborn on the other hand ventilation decreased succesively after vago tomy (Fig 5 C) whereby the arterial pCO_2 rose while the pH decreased (Fig 5 C) without apparently giving rise to any effective stimulation of respiration Such a stimulation might have occurred initially (Fig 5 C 10 min) but was ineffective in maintaining an adequate ventilation since CO_2 continued to accu mulate From Fig 5 D it can be seen that the blood pressure remained fairly con stant during the experiment Similar results were obtained in all 6 animals below 2 weeks of age that were tested in this respect irrespective of whether they were decerebrate or anesthetized

In conclusion it can be stated that whereas ventilation was still sufficient to keep the arterial pCO_2 and pH constant during at least the first hour after vago tomy in 4—5 months old cats this was not the case in kittens below 2 weeks of

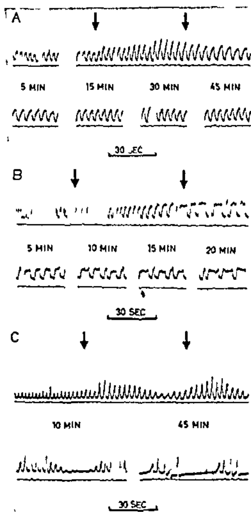


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of the common carotid arteries and the interruption of the posterior communicating arteries intracranially on decerebration Direct inspection also revealed that the carotid arteries were well filled with bright red blood at the level of the bifurcation

Discussion

The principal findings were that ventilation in animals below 2—3 weeks of age decreased after vagotomy and that the arterial pCO_2 and hydrogen ion concentration increased It could be argued that a change in pressure amplitude might not reflect accurately the ventilation for two reasons both of which however will result in an underestimate of the degree by which it is depressed If pulmonary edema develops within the relatively short experimental period used the pulmonary compliance will actually decrease (see Radford 1964) and the pressure amplitude will thus give a relative overestimate of the inspired volumes Secondly since the volume pressure curve bends slightly to the right at the upper extreme of vital capacity (see Radford 1964) the pronounced increase in pressure that was sometimes seen after vagotomy might also result in an overestimate of the ventilation A further confirmation that there is a true ventilatory decrease in the youngest animals after vagotomy was the finding of an increase in arterial pCO_2 It is generally accepted that the pCO_2 reflects the adequacy of the alveolar ventilation (Severinghaus 1965) It cannot be excluded that the accumulation of CO_2 is in part due to the development of a pulmonary edema and a consequent decrease in respiratory surface but if so it is still noteworthy that the newly born is unable to increase ventilation in response to an increase in arterial pCO_2 after vagotomy

As pointed out in the Introduction the vagal nerves of the adult contain afferents of which some inhibit and other facilitate respiration The finding that vagotomy prolonged the inspiratory phases in the newborn animal indicates that the inhibitory afferents are already functioning at that stage Moreover this inhibition appeared to be relatively more important in the newborn since respiration sometimes became gasp like with very long expiratory pauses (Fig 5 D) or periodic (Fig 4 C) The periodic breathing was similar to Biot s" or Cheyne—Stoke s respiration which in the adult can be produced by different brain stem sections (see Hoff and Beckenbridge 1954) and which reflects incoordination of the respiratory feed back system Possible reasons for these reactions will be taken up in the General Discussion If now the vagal inhibitory afferents are of such a pronounced importance in the newly born it seems justifiable to assume that they are relatively mature already at birth This will be directly studied in Chapter V

It appears likely however that the ventilatory diminution after vagotomy was also due to the withdrawal of some facilitation Such a facilitation might be exerted by the aortic and the pulmonary chemoreceptors The results of the

subsequent denervation of the third set of such receptors viz those situated in the carotid glomus actually supports this assumption when following vagotomy also the sinus nerves were sectioned respiration immediately decreased further and often rapidly failed There is some previous evidence that the peripheral chemoreceptors are functioning in the newly born (see Chapter I) However direct studies on this problem are extremely sparse especially with regard to the kitten and this matter is treated further in Chapter VI

It must be pointed out that the vagal nerves contain the afferents of two other kinds of pulmonary receptors which on stimulation exert a facilitation on the respiratory center Firstly there are those from the so called deflation receptors (Paintal 1955 1957) which fire in response to a more or less pronounced collapse of the lungs The reflex facilitation of respiration which is assumed to be set up by them—the Hering—Breuer deflation reflex"—has been shown to be present at birth in rabbits (Dawes and Mott 1959) However very little is known about the physiological significance of this reflex and it is generally held that it is without importance during normal breathing in the adult (see Widdicombe 1964)

Secondly there is a set of receptors giving rise to the so called Head s "paradoxical reflex which consists of an inspiratory effort in response to lung inflation (Head 1889) In the newborn infant and lamb this reflex is very easy to produce (Cross Klaus Tooley and Weisser 1960 Hughes Parker and Williams 1967) whereas in the adult a partial vagal block by cooling to some 10°C is necessary No conclusive evidence has been presented however as regards the receptors responsible for this reflex (Paintal 1966 Widdicombe 1967)

The problem now remains as to whether the decrease in ventilation after vagotomy can be attributed to the interruption of nervous pathways other than those directly concerned with respiratory control e g circulatory or other visceral pathways With regard to the circulation it was found that the blood pressure remained reasonably constant during at least the first hour after vagotomy (Fig 5 D) suggesting that circulatory disturbances are not responsible for the ventilatory decrease Whether the interruption of other visceral reflexes has any direct relation to the respiratory decrease is more difficult to evaluate Nothing seems to be known for example about the possibility that vagotomy affects the behaviour of electrolytes in the newborn kidney (see Cort 1965) Such possible changes might however be expected to occur more slowly and to be of minor importance in these acute experiments

Summarizing briefly it appears that the respiratory failure after vagotomy is in part due to the withdrawal of inhibition whereby the respiratory frequency is reduced This eventually leads to respiratory failure probably also because of withdrawal of facilitation from peripheral chemoreceptors and from some of the pulmonary mechanoreceptors viz those responsible for the paradoxical reflex" of Head

The next question is whether some other receptor systems concerned with respi ratory regulation are not functionally mature at birth which might contribute to the explanation for the deleterious effect of vagotomy in the newborn As pointed out in Chapter I the thoracic dorsal roots have recently been shown to be of great importance for respiratory control in the adult (see Euler 1966) Since very little appears to be known about this control system in the newborn or during postnatal development it will be treated in Chapter VII

Furthermore in the present work it was found that the increase in CO_2 of the blood after vagotomy was ineffective in stimulating ventilation (Fig 5) This of course further adds to the deleterious effect of vagotomy in the newborn and raises the question whether the central chemosensitive mechanism, which is known to respond primarily to changes in acid base values of the blood and/or medullary extracellular or cerebrospinal fluid (see Chapter I) is functionally developed at birth This problem will be treated in Chapter VIII

It might be pointed out that animals above 4 weeks or some 30 days of age were found in general to respond to vagotomy as the adult Coombs and Pike (1930) on the other hand reported that this stage was not reached until 40—50 days after birth However these authors only studied the survival time which also includes the intrapulmonary changes (see *Introduction*)

Summary

1) The effect of section of the vago sympathetic trunks and the sinus nerves on respiration has been studied in cats and rabbits at different ages from the newborn to the adult Anesthetized and decerebrate animals were used In addition to respiration also arterial pCO_2 and pH and blood pressure were continuously recorded in some cats and kittens

2) On section of the cervical vago sympathetic trunks

a) the inspiratory phase of respiration becomes prolonged in both newborn and adult cats and rabbits However the respiratory pattern is more affected by the vagotomy in animals below 3 weeks than in those above 4 weeks of age This is especially marked in decerebrate animals where respiration often becomes periodic or gasp like

b) In both decerebrate and anesthetized kittens below 2 weeks of age ventilation successively diminished with a consequent increase in arterial pCO_2 which does not give rise to any effective ventilatory stimulation This is not the case in adult animals where arterial pCO_2 and pH remain constant

3) When the carotid chemoreceptors are denervated by section of the sinus nerves following vagotomy respiration decreases only slightly in animals above 4 weeks whereas it is strongly depressed and rapidly fails in animals below 3 week of age

The physiology and morphology of the pulmonary tension receptor afferents during postnatal development in the cat

Introduction

In this chapter the afferents responsible for the "Hering Breuer inflation" reflex will be studied The first question to raise is Is this reflex present at birth in the kitten? The pulmonary tension receptor afferents will then be directly studied and the following questions treated What is the normal discharge pattern of these afferents at birth? Does this discharge pattern change postnatally? Are the pulmonary tension receptors able to fire continually on a maintained stimulus already at birth? Is the correlation between functional and morphological maturation of the pulmonary tension receptor afferents the same as that found in other nerves (as for example the sural nerve see Ekholm 1967) during postnatal development?

The recordings will be made from fibres in the cervical vagus nerves These nerves contain a vast number of fibres other than those from the lungs why the morphological analysis will be made on a bronchial nerve Since the anatomy of the bronchial nerves is poorly known in the cat a short anatomical description of them will be given

Material and Methods

The Hering—Breuer inflation" reflex was tested in a very large number of cats of all ages (both anesthetized and decerebrated) that were used also for other purposes Recordings from vagal afferents are presented from 10 experiments carried out on 6 kittens aged 1—3 days 2 kittens aged 7—10 days 1 cat aged 120 days and 1 adult cat all lightly anesthetized with Nembutal The trachea was cannulated and the vagi exposed in the neck One vagus nerve was

sectioned just below the nodose ganglion the sheath was removed and the nerve teased into finer and finer filaments from which the recordings were made under paraffin oil at 37°C A thin silver hook was used as the active recording electrode a reference electrode being inserted into inactive tissue The spike potentials were recorded between these two electrodes and fed via a cathode follower into an amplifier and displayed on a cathod ray tube Time was indicated by a sine wave generator or a piezo crystal on a second beam The discharge was also monitored by a loudspeaker

The anatomical distribution of the vagal bronchial nerves was studied in 9 cats The animals were killed by an overdose of Nembutal a wide thoracotomy was performed and the dorsal part of the thoracic cage was together with the thoracic viscera removed from the animal and immersed in 1 % acetic acid for 5 to 10 hours This procedure fascilitates dissection which was carried out under water *with the aid of a dissecting microscope*

The fibre calibre spectra were determined on the right bronchial and sural nerves of 4 kittens aged 2 6 35 and 45 days and one adult cat The histochemical test of Berthold and Skoglund (1967) for $NADH_2$ tetrazolium reductase was also made on these nerves and on the same nerves of five additional newborn kittens (age 1—4 days) The nerves were exposed by careful dissection (the sural nerve at the lower border of the popliteal fossa the bronchial nerve (see p 42) in its whole length) and fixed in situ for 10 minutes with a 5 % glutaraldehyde solution (see below) The nerves were then excised and in the case of those *destined for fibre analysis cut into two pieces The fibre" piece was fixed in* the glutaraldehyde solution for another 4 hours the piece used for histochemical analysis for another 20 minutes This latter was then teased carefully under a dissecting microscope and incubated at 37°C for 30 minutes in a medium containing $NADH_2$ as substrate and Nitro BT as reagent (see Berthold and Skoglund 1967)

Fibre calibre analyser The nerve specimens were fixed in a 5 % glutaraldehyde solution dissolved in a phosphate buffer (Karlsson and Schultz 1965) The tonicity of this solution was cryoscopically measured to be about 600 milliosmols (glutaraldehyde 550 phosphate buffer 50) The specimens were then *rinsed in* icecold veronal buffer (for details see Berthold 1968) for 4 hours postfixed in 2 % OsO_4 (in the veronal buffer) for 4 hours dehydrated in a graded series of acetone and embedded in Vestopal W (Ryter and Kellenberg 1958) and cut on a LKB 4800 ultrotome in 0 5 μ thick sections These were then stained with toloudin blue photographed on rollfilm at a linear magnification of 120—320 and enlarged to an overall linear magnification of 1000 or 2000 The linear magnification was always controlled by including in one field a scale with 10 μ divisions The diameters of all the myelinated nerve fibres were then measured with a particle size analyzing machine (Zeiss TGZ 3) according to the *method*

of Romero and Skoglund (1965)

The reason for choosing glutaraldehyde fixed and Vestopal embedded material for the calibre analysis was that the quality of these preparations was superior to that obtained with the classical method of fixation in OsO_4 followed by paraffin embedding In fact with this latter method it was found impossible to obtain useful preparations of the bronchial nerve from newborn animals The disadvantage with the glutaraldehyde fixation on the other hand is that another shrinkage factor must be expected which makes direct comparisons with the material from Skoglund and Romero (1965) which includes the sural nerve impossible Thus the calibre spectra of the sural and the bronchial nerves were both determined to allow direct comparisons between them

The reason for choosing a 5 % glutaraldehyde in a 50 milliosmolal vehicle as the fixation solution was to obtain fibres as round as possible in order to be able to use the particle size analyzing machine which is of considerable advantage when measuring nerve fibre diameters (see Romero and Skoglund 1965) Berthold (1968) has shown that the configuration of nerve fibres in cross section is dependant on the fixation solution used Thus a series of different fixation methods have been tried in order to obtain fibres as round as possible without disturbing the quality of the preparation This methodological study will be presented in full elsewhere (Berthold and Schwieler to be published) Suffice it to say here that the 5 % glutaraldehyde solution gave acceptable preparations

Fig 7 shows part of a cross section from a sural nerve from a 2 days old kitten at a linear magnification of 2000 The nerve had been treated as described above and is representative for the material used It can be seen that some fibres are oval or more or less irregular shaped and the method of measuring was first studied in some detail A series of some 100 serial cross sections was made from

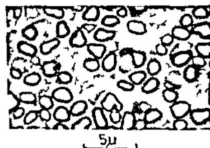


Fig 7 Cross section from part of the right sural nerve from a days old kitten at linear magnification of 2 000

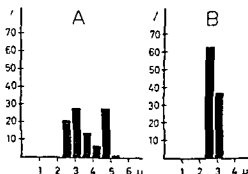


Fig 8 Histograms of the diameters obtained from measuring one and the same nerve fibre on 100 consecutive serial sections with two different methods (for further explanation see text)

the same sural nerve as in Fig 7 One fibre was then followed throughout the series of sections and measured in two ways First the diameter of the circumscribing circle was measured and the result is shown in Fig 8 A As can be seen the diameter of the same fibre was found to vary between 2 5 μ and 5 5 μ which is unacceptable but it is perhaps not unexpected as the shape is highly irregular in the paranodal region (see Be thold and Skoglund 1968) The same fibre was then measured throughout the series as illustrated in Fig 9 if the fibre was triangular in shape (A) a circle was chosen which included most of the nerve but left the extensions outside If the fibre was oval (B) the longest minor axis was measured unless this diameter was smaller than half of the "longest" major axis in which case it was omitted If the fibre was clover shaped (C) finally it was again not measured The result is shown in Fig 8 B As is seen with this method the diameter varies much less It may be questioned whether exclusion of some values was a valid pro edure but a high deg ee of ellipsoidal eccentricity probably means that the fib e is cut obliquely clover shaped fibres were p obably from the paranodal region where the diameter is not always representative (see Berthold and Skoglund 1968)

As a next step all myelinated fibres in four of the sections at approximately 10 μ intervals were then measured with the particle size analyzing machine and the results are shown in Fig 10 The total number of fibres varied between 612 and 646 and as is seen from the histograms the diameter distributions are almost the same

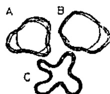


Fig 9 Diagram to show how nerve fibres not being circular were measured (see text)

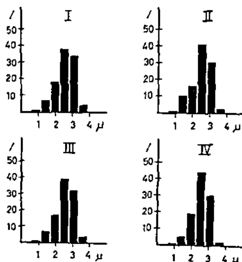


Fig 10 Histograms of the calibre spectra from four different cross sections 10 μ apart, of the right sural nerve in a 2 days old kitten

I *The Hering Breuer inflation reflex*

This reflex was tested simply by occluding the tracheal cannula at the height of inspiration until the next maximal pressure rise whereby the lungs are prevented from collapsing and the discharge from the pulmonary tension receptors thereby maintained (see below section II) At all ages the response was a prolongation of the expiratory pause as shown in Fig 11 from a newborn kitten which was never seen after bilateral vagotomy Thus as in other species (see Chapter I) the Hering Breuer inflation" reflex is present at birth in kittens and the vagal afferents from the pulmonary tension receptors appear functionally mature enough to affect the respiratory center in this respect as in the adult No attempt was made to test if there are any quantitative differences between the newborn and adult

II *The discharge pattern of single pulmonary tension receptors in cats of different ages*

Action potentials were recorded from single nerve fibres in the peripheral end of one cut cervical vagus nerve (the contralateral nerve being intact) Those fibres which increased their discharge frequency in parallel with inspiration are assumed to be afferents from pulmonary stretch receptors (Adrian 1933) All animals were prone and were breathing room air

a) Cats above 4 months of age

For comparison the discharge pattern of the pulmonary stretch receptors was analyzed in adult cats In confirmation of earlier findings a very broad frequency spectrum was found although two main types of receptors were identified one firing continually during the whole respiratory cycle "low threshold receptor" (Paintal 1966) and one firing only during inspiration high threshold receptor" (Paintal 1966) Of some 50 fibres analyzed in 2 animals the ratio between "high" and "low threshold" receptors was 1 1 (Tab II) which is in agreement with Paintal s findings The maximal frequency in different high threshold" receptors

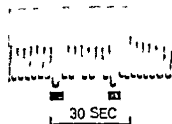


Fig 11 Respiratory record (intraoesophageal pressure swings inspiration deflection upwards) from a 1 day old kitten anesthetized with 30 mg/kg Nembutal The tracheal cannula was occluded at the height of an inspiration until the next maximal pressure rise as indicated As can be seen, the expiratory paus was thereby prolonged

Table II Number af high and low threshold receptors found when recording from single vagal pulmonary afferents in cats of different ages

Age in days	Low threshold	High threshold	Total
1 1 1 1 3	0	43	43
7 10	6	20	26
120 adult	22	25	47

at the peak of a normal inspiration varied between 40 and 80/sec The frequency of low threshold" receptors ranged between 20 and 60/sec during the expiratory paus and increased to some 100—140/sec during inspiration These two kinds of receptor discharges are illustrated in Fig 12

b) Kittens below 2 weeks of age

Out of some 40 receptors analyzed in 6 kittens aged 1—3 days none was found to be of the low threshold" type described above (Tab II) The maximal frequency at the peak of inspiration varied between 20 and 50/sec. in different receptors (Fig 12)

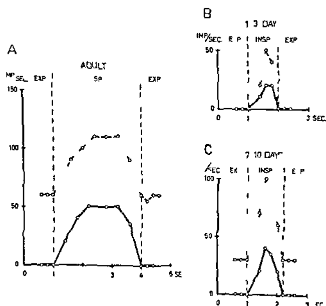


Fig 12 Frequency curves of the discharge in different single vagal afferent nerve fibres firing synchronously with respiration in cats of different ages The animals had been anes thetized with 30—40 mg/kg Nembutal were prone and breathed room air One vagus nerve was intact

It may be asked if the absence of "low threshold" receptors was due to an inability of the receptors to fire continually This was tested by occluding the trachea at the height of inspiration while recording from a single pulmonary strech unit It was a constant finding that the firing continued after a slight decrease in frequency for more than 3 minutes (Fig 13)

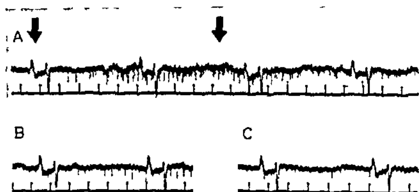


Fig 13 1 day old kitten Recording from a thin filament of the left cervical vagus nerve The first arrow indicates beginning of inspiration At the second arrow (height of inspiration) the tracheal cannula was occluded B 1 min C 3 min after the onset of occlusion Interference from ECG on the upper beam Time 100 msec

Finally some 20 units were analyzed in 2 kittens 7—10 days of age The ratio between "low" and "high threshold" receptors was 3 10 (Tab II) and the frequency ranges during a respiratory cycle for these receptors 20—100 and 0—50/sec respectively (Fig 12)

In conclusion then the normal discharge range of the pulmonary tension receptors is smaller and thus the maximal frequency during inspiration lower in newborn kittens However the receptors posses the ability to fire continually on a maintained stimulus

III *Anatomy of the vagal bronchial branches in the cat*

Since the vagal nerves from which the recordings were made contain a vast number of fibres which do not originate from the lungs it was decided to make the morphological investigation on some pure pulmonary nerve Agostoni Chinock De Burgh Daly and Murray (1957) and Paintal (1963) have shown that the biggest fibres in the thoracic vagal bronchial rami are afferents from the pulmonary tension receptors The anatomy of these rami is however poorly described in the literature According to Stowell (1882) and Reighard and Jennings (1961) the vagal nerves give off a number of branches at the level of the root of the lungs which form a cardiac and a pulmonary plexus From these plexuses

numerous branches are described to pass to the heart lungs pulmonary artery pericardium etc It has been shown that in most mammals (for reference see Hayek 1953) the nerves leaving the pulmonary plexus form three main groups one for the vessels one for the bronchial tree and one for the visceral pleura As the pulmonary tension receptors studied appear to be located in the bronchial walls (Widdicombe 1954) it was desirable to choose a nerve branch solely destined for the bronchi and which had a reasonably constant course in different cats An anatomical study of the distribution of the pulmonary vagal branches was therefore made in 9 cats

Each vagus nerve was found to give off 4—5 larger branches and a number of smaller branches at the level of the lung hilus (Fig 14) The largest branches are directed ventrally and the smaller both dorsally and ventrally in relation to the hilus The most cranial of the larger branches constantly leave the vagus nerves at the level of the azygos vein on the right and at the level of the aortic arch on the left side These branches (I) are distributed to the pericardium the heart and especially to the pulmonary trunk and corresponds to the branches which according to Krahl (1962) supply the pulmonary glomus From these nerves

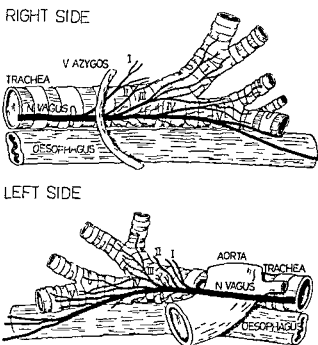


Fig 14 Schematic drawing showing the principal courses of the branches leaving the vagi at the level of the lung hilus Nrs I and II are mainly distributed to the heart and pulmonary trunks nrs III—V to the main bronchi

and/or from the succeeding branches (II) small twigs are distributed to the visceral pleura of the lungs and also to the pulmonary vessels Then follow 2—3 (III—V) additional relatively large branches which are all destined for the main bronchi Of these the first two (III and IV) were usually larger than the others and sometimes had a common origin from the vagus nerve

In addition there are a lot of smaller branches varying in number which spread diffusely to the heart vessels and bronchi Some of them are directed dorsally towards the lung hilus and anastomose with branches from the contra lateral vagus From the plexus thereby formed nerve twigs are given off to the bronchi and to the oesophagus

The distribution of the branches varied considerably from animal to animal especially as regards the smaller ones In the study of fibre composition and histochemistry which follows No III and when having a common origin No IV on the right side were taken since they could be followed peripherally coursing

Table III The calibre spectrum of r bronchialis

Age in days	Animal nr	0 66 1 21	1 21 1 76	1 76 2 3	2 3- 2 87	87 3.4	3- 3
2	KT 39	10	41	7	45	1?	
6	KT 25	6	47	40	43	1?	
35	KT 43		1	20	38	57	1
adult	KT 42		7	68	43	29	?

Table IV The calibre spectrum of n suralis

Age in days	Animal nr	0 66- 1 21	1 21 1 76	1 76 2 3-	2 32 2.87	2 87 3.4-	3 4 3 97	3 97 -4.52	4 5- 5 08	5 08 5 63	5.63 -6 18	6 18 -6 73	6
2	KT 39	5	15	11	53	05	6						
6	KT 5	5	01	101	177	179	83	29	9	1	1		
35	KT 43		6	91	135	11	115	107	103	15	14	7	
adult	KT 4?		4	11	1(	91	116	131	68	18	2	3	

out on the main bronchi In order to ensure that they contained afferents from the tension receptors recording was made from the whole cervical vagus on a thoracotomized animal artificially ventilated A distinct discharge which was synchronous with inflation could be heard in the loudspeaker After sectioning branches III and IV most of this discharge disappeared immediately which indicates that they do contain afferents from the pulmonary tension receptors

IV *The fibre calibre spectra of the bronchial and sural nerves and the paranodal $NADH_2$ tetrazolium reductase activity in bronchial and sural nerve fibres*

Tables III and IV and Fig 15 show the calibre spectra and histograms of the bronchial and the sural nerves respectively Since the nerves were always taken from the same animal direct comparisons between them are possible In the newborn the nerves show a similar fibre composition most fibres being between 1 μ and 3 μ with a slight dominance of larger fibres in the sural nerve The largest fibres were in both cases between 3 5 μ and 4 μ However the

4 52 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 8	7 28 7 84	7 84 -8 39	Numb r of fibres measured
							184
							15?
5							183
4	29	15	8	6	3	1	292

7 84 8 39	8 39 8 94	8 94 9 49	9 49 10 04	10 04 10 60	10 60 11 15	11 15 11 70	11 70 1. .5	12 .5- 12 80	1 80- 13 .6	Number of fibres measured
										646
										705
										765
50	61	84	80	57	5(	24	6	11	8	1092

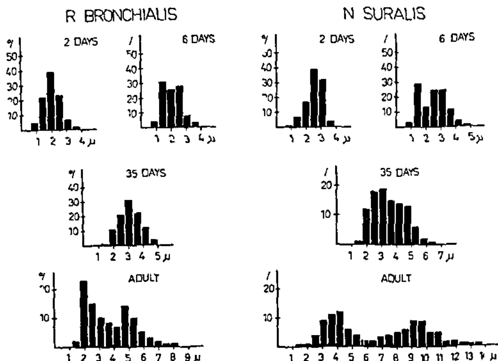


Fig 15 Histograms of calibre spectra of the bronchial and sural nerves in cats of different ages given in the figure Note different scales on the ordinates

postnatal growth of the sural nerve fibres is much more pronounced In the adult stage the largest fibres of this nerve have attained some 14 μ and there is a bimodal distribution with one peak at 4—5 μ and the other at about 10 μ The largest bronchial nerve fibres in the adult stage on the other hand are only around 8 μ and the two peaks are at about 2 0 μ and 5 μ Thus as judged from their postnatal growth the bronchial nerve fibres are relatively better developed at birth since their postnatal growth is much less

Also with regard to the paranodal NADH_ reductase activity many bronchial fibres showed a considerably more mature pattern than the sural ones at the newborn stage Berthold and Skoglund (1967) classified the enzyme activity of the fibres into three types and in accordance with this classification the sural nerves from the newborn and 6 days old kittens contained types 1 and 2 fibres only (Fig 16 A—C) of which the former was most frequent In the bronchial nerves however type 3 fibres were also frequently found (Fig 16E—G) Berthold and Skoglund found type 3 predominantly among large fibres of older animals but here very small fibres could also be classified as type 3 (Fig 16 G) whereas fibres of comparable or even larger size of the sural nerve always belonged to type 1 In the nerves from the 35 and 45 days old animals most fibres were of type 3 and showed the adult staining pattern (Fig 16 D H)

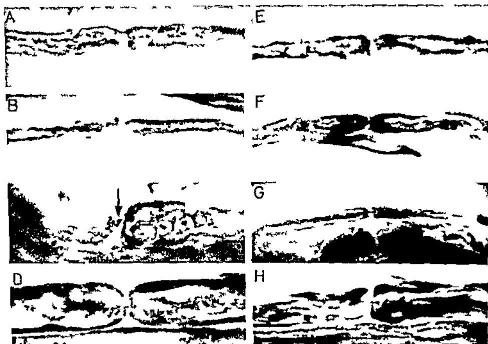


Fig 16 Light microphotographs of teased nerve fibres fixed in glutaraldehyde and stained for NADH tetrazolium reductase activity A—C and E—G sural and bronchial fibres respectively from the same ‿ days old kitten D H sural and bronchial fibres from a 35 days old kitten A B type 1 fibres Reductase pattern spread diffusely over the fibres No accentuated paranodal stripes C type ? fibres with characteristic network of reductase activity and assymetry between the two paranodal pairs (Arrow indicates node gap) D—H type 3 fibres Marked internodal bands with accentuated staining at the paranodal region Note the small size of the bronchial fibre in G All photos X 1380

It may thus be concluded that the fibre size compositions of the bronchial and sural nerves are similar in the newborn but that since the most mature stage of enzyme activity (type 3) is found in smaller fibres in the bronchial nerve this nerve appears to be at a more advanced stage of maturation than the sural nerve This appears to be reflected in a smaller postnatal growth of the bronchial fibres

Discussion

The present work shows that the Hering Breuer inflation" reflex is present in the newborn kitten as it is in other species (see Chapter I) The receptors generally held to be responsible for the reflex viz the slowly adapting pulmonary tension receptors (Adrian 1933) were found to discharge with essentially the same pattern as in the adult although the frequency range was smaller Thus the maximal frequency was found to be lower (about 50/sec. as compared to

140/sec in the adult) and no receptors fired continually i e also during the expiratory pause Since the method of recording probably has a tendency to select the biggest fibres i e probably the most mature ones (see Skoglund 1966 Berthold and Skoglund 1967 Ekholm 1967) the finding of lower maximal frequencies appears to be significant The same must be expected to hold true also for the apparent absence of "low threshold" receptors it seems highly unlikely that this is due to a selection of small and immature fibres Moreover when tested the receptors were found to be able to discharge continually when the lungs were kept distended

The finding that the discharge frequencies from the pulmonary tension receptors are generally lower in the newborn might reflect different mechanical properties of the lungs and chest wall at birth for example the lungs appear to be relatively more collapsed at expiration i e the functional residual capacity (the volume of the gas in the lungs and airways at the end of a spontaneous expiration) is relatively smaller in the newborn (see Agostoni and Mead 1964) This might partly explain why no "low threshold" receptors were found in the newborn kittens since "high" and "low" threshold receptors are apparently of the same kind but under different degrees of distension Thus in the adult a "low" threshold receptor can be changed into a "high" threshold receptor if the distension is decreased for example by increasing the depth of the expirations (Adrian 1933)

Of course the receptors themselves might undergo functional changes during growth but the explanation for the lower frequencies might also be that the afferent nerve fibres are *relatively* immature (cf Skoglund 1966 Ekholm 1967) However in comparison with the sural nerve fibres those of the bronchial nerve appear to be better developed physiologically in that they are able to transmit impulses continually

This ability of the receptors to fire tonically is in striking contrast to the case with different somatic receptors which in the newborn kitten are able to discharge phasically only in response to a maintained stimulus (see Skoglund 1960 c Ekholm 1967) When recording from touch units in the sural nerve Ekholm (1967) found that with a constant pressure applied to an adequate skin area there was only a discharge of short duration Not until the age of 2—3 weeks postnatally were tonic discharges observed Skoglund (1966) postultated that the reason for the inability to fire continually is to be sought in the afferent nerve fibres and not in the receptors themselves (see Chapter I) Ekholm (1967) found positive evidence for this idea in experiments where he stimulated through the skin electrically in the receptive field in an attempt to bypass the receptors

In the present work the fibre composition of the bronchial nerve in newborn animals was found to be similar to that of the sural nerve but the bronchial fibres were found to grow much less postnatally which further indicates that

they are relatively more developed at birth This was also supported by the finding that the bronchial nerve fibres had a more mature paranodal $NADH_2$ reductase pattern in relation to their size than sural fibres Thus it appears that the stage of maturity of a nerve fibre that can be revealed histochemically i e the paranodal $NADH_2$ reductase activity is a better correlate to functional maturity than fibre size as suggested by Berthold and Skoglund (1967) This will be further discussed in Chapter VI

It must be pointed out that the results obtained from the comparisons between the functional and morphological properties of the nerve fibres may be only valid for the biggest fibres in recording action potentials there may be a marked tendency when teasing out single fibres to preserve the larger ones Then if the reason for the finding of lower frequencies is the relative immaturity of the afferent nerve fibres it must be excepted that those being less mature morphologically are also less mature physiologically

It is noteworthy that in spite of the low discharge frequencies the pulmonary tension receptors were found to affect respiration as in the adult Cross and co workers (Cross Klaus Tooley and Weisser 1960) even found that the Hering—Breuer inflation" reflex was stronger in the newborn infant than in adult man Wyss (1964) has repeatedly argued that the pulmonary tension receptors facilitate inspiration when firing at low frequencies (less than 30/sec) but inhibit inspiration at higher frequencies If this is right and holds true for the newborn the receptors should facilitate inspiration during a relatively longer period However the results on which Wyss (1964) bases his hypothesis is not convincing since he studied the effect of artificial stimulation of the afferent vagus nerves or simply of vagotomy When stimulating the afferent vagi artifically it is impossible to exclude that other receptor afferents than those which inhibit respiration are affected The same holds true for the vagotomy when the vagi are sectioned different afferent systems are cut which might both facilitate and inhibit inspiration (see Discussion Chapter IV)

The relative importance of the pulmonary tension receptors for respiratory control in the newborn will be taken up in the General Discussion

Summary

1) The Hering—Breuer inflation" reflex was found to be present in the newborn kitten since tracheal occlusion at the height of an inspiration prolonged the expiratory phase

2) The discharge pattern from the pulmonary tension receptors which are responsible for the Hering Breuer inflation" reflex was analysed The receptors were found to fire with lower frequencies at birth but to be able to fire continually in response to maintained distension of the lungs

3) The anatomy of vagal bronchial nerves is described

4) The calibre spectra and paranodal $NADH_2$ tetrazolium reductase pattern of single nerve fibres were analysed in the bronchial and sural nerves during postnatal development Although the calibre spectra of the bronchial and sural fibres were similar at birth the bronchial fibres grow much less postnatally and exhibit a more mature paranodal reductase pattern in relation to their size in the newborn

5) Possible reasons for the finding of lower discharge frequencies of the pulmonary receptors are discussed

The physiology and morphology of the peripheral chemoreceptor afferents and the ventilatory reactions to changes in oxygen concentration of the respired air during postnatal development in cats and rabbits

Introduction

In this chapter firstly recordings will be made from the carotid chemoreceptor afferents in kittens breathing air containing different concentrations of oxygen. Then the calibre composition and histochemistry of the sinus nerve fibres will be studied in order to correlate functional and morphological properties in these afferents as was done for the bronchial fibres in Chapter V. The first questions to be treated are: Are the carotid chemoreceptors able to fire continually in the newborn animals and to respond by changing their discharge frequencies to changes in the oxygen concentration of the respired air? Is the correlation between the functional and morphological maturation of the sinus nerve afferents the same as that found in other nerves?

Against the background of the thus acquired knowledge the ventilatory reactions to changes in oxygen concentration of the respired air will be investigated to further elucidate to what an extent the peripheral chemoreceptors are of importance for the regulation of breathing in the newly born and during postnatal development. Besides respiration also the arterial pH and pCO_2 will be recorded in some cases. The following questions will be raised: Are the peripheral chemoreceptors continually facilitating respiration during air breathing in the newborn animal? Is the secondary ventilatory depression in response to hypoxia in newborn animals due to a decrease or cessation of the peripheral chemoreceptor discharge? Do any changes in arterial pCO_2 and pH occur during hypoxia in newborn animals?

Material and Methods

Recordings from the carotid chemoreceptor afferents were made on 4 kittens aged 1—2 days 5 kittens aged 3—6 days and 3 kittens aged 13—19 days All the 12 animals were lightly anesthetized with Nembutal and a cannula was inserted into the trachea The right sinus nerve (ramus sinus carotici) was exposed by careful dissection from a midline incision in the neck The carotid bifurcation was indentified and the approach made medial to it A binocular microscope (Zeiss) was used during the subsequent dissection In the bottom of the wound medial to the carotid bifurcation is the tympanic bulla traversed by the glossopharyngeal nerve as it passes from the jugular foramen in an antero lateral direction As a rule the sinus nerve enters the glossopharyngeal nerve as it lies on the bulla The former could then easily be traced to its origin in the carotid glomus The whole sinus nerve in a newborn kitten is less than 2 mm long and very thin In order to prevent it from drying paraffin oil (38°C) was poured into the wound The sinus nerve was then cut at its entrance into the glossopharyngeal nerve and carefully dissected free from the surrounding connective tissue The sheath was removed and the nerve teased into finer and finer filaments from which recordings were made The spike potentials were recorded with the nerve under paraffin oil with the system described in Chapter V (p 35) The recordings were made when the animal was breathing room air (21 % O_2) 10 % O_2 in N_2 or 100 % O_2 the gases were administred as described on page 21

The fibre calibre spectra and the paranodal $NADH_2$ tetrazolium reductase activity were studied in sinus nerves taken from the same animals as the bronchial and sural nerves in the experiments described in Chapter V the sinus nerves were treated in exactly the same way (p 35)

The ventilatory reactions to changes in oxygen concentration of the respired air were studied in 16 experiments carried out on cats and rabbits of different ages ([illegible] kittens 1 [illegible] 7 days 9 kittens 8—19 days 4 kittens 28—35 days 3 adult cats and 13 rabbits 1 [illegible] 4 days 4 rabbits 21 days 4 rabbits 8 weeks—adult) Most of the cats and kittens were lightly anesthetized with Nembutal while most of the rabbits were anesthetized with urethane A few kittens and rabbits were decerebrated (p 18) The trachea was cannulated and the vagus and sinus nerves exposed Respiration was recorded as the intraoesophageal pressure swings (p 18) In a few cases also the arterial pH and pCO_2 were recorded continuously as described previously (p 20)

Results

1 *The [illegible] carotid chemoreceptors in newborn kittens breathing [illegible]*

In Fig 1 [illegible] records from the discharge of a thin filament of the right

carotid sinus nerve (the left nerve being intact) in a newborn kitten breathing gas mixtures with different concentrations of oxygen As may be seen the records also include the discharge from the carotid sinus pressoreceptors It is evident that the chemoreceptors are firing continually during airbreathing a discharge which decreases when air is replaced by 100 % oxygen and which increases considerably and more or less fills in the intervals between the pressoreceptor dis charges in response to hypoxia (10 % O) as it does in the adult (see Hey mans and Neil 1958) This discharge was always found to continue unaltered for several minutes or increase slightly but never showed any sign of decreasing in spite of the fact that ventilation decreased or even failed (see below)

To ensure that the single receptors are able to fire continually (the possibility exists that different receptors discharge alternately) recordings from single chemoreceptor afferents were also made Fig 18 shows the records from such an experiment The receptor is seen firing tonically with a frequency between 5 and 10/sec when the animal breathes air and with about 20 /sec when it is breathing 10 % O in N Due to the extreme difficulties in obtaining single fibre pre parations in these small nerves no systematic frequency analysis has been made Of 6 single units analysed in 4 kittens aged 1—2 days however all showed a continuous discharge and a sustained increase in frequency in response to a de crease in oxygen content of the respired air

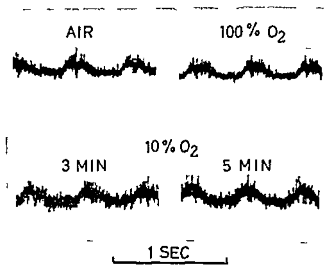


Fig 17 Re ordings from a thin filament of the right sinus nerve in a 1 day old kitten breathing room air pure oxygen and 10 % O in N_2 (3 and 5 min indicates time after beg nning of administration of 10 % O_2)

Table V The calibre spectrum of r sinus carotici

Age in days	Animal nr	0.66 1.1	1.21 1.76	1.76 2.32	2.32 2.8
2	KT 39	25	217	160	
6	KT _5	20	185	166	3
35	KT 43	7	100	2[illegible]4	8
adult	KT 4?		48	206	15

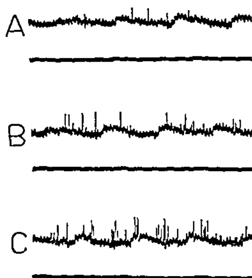


Fig 18 Recordings from a thin filament of the right sinus nerve in a 4 days old kitten breathing pure oxygen (A) room air (B) and 10 % O_2 in N_2 (C) Note in (C) at least one new unit is seen firing Time 100 msec

II *The fibre calibre spectrum of the sinus nerve and the paranodal NADH tetrazolium reductase activity in sinus nerve fibres in the cat*

The calibre spectrum and histograms of the sinus nerve are shown in Table V and Fig 19 respectively It can be seen that in the nerves from the youngest animals most of the fibres are between 1 μ and 2 μ in diameter and that very few fibres are above 3 μ the largest fibres being about 3 5 μ During postnatal development the peak value moves only slightly to the right and in the adult animal most of the fibres are between 2 0 μ and 3 5 μ and the largest fibres are only about 7 μ in diameter Thus compared with the bronchial and sural nerves (cf Chapter V) which were taken from the same animals both the size of the biggest fibres and of the fibres comprising the peak is smaller in the newborn sinus nerve However the sinus nerve fibres grow relatively much less post natally and with regard to fibre size they seem in fact to be even better developed

87 :42	3 4 3 97	3 97 -4.5..	4 5.. 5 08	5 08 5 63	Number of fibres m asured
5					4?8
10	2				417
12	12	1			479
57	15	7	6	3	507

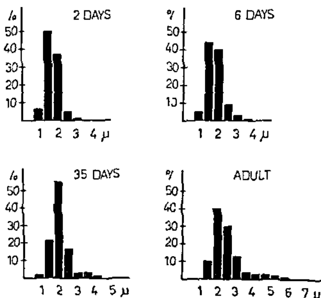


Fig 19 Histograms of calibre spectra of the s nus nerve in cats of different ages given in the figure

at birth than the bronchial nerve fibres Moreover the total number of fibres does not increase as much as for example in the sural nerve

The paranodal reductase activity pattern in the sinus nerve fibres was very similar to that of the bronchial fibres (cf Chapter V) of the same animals Thus besides type 1 and 2 fibres type 3 fibres were found frequently (Fig 20 A B C) in the 2 and 6 days old kittens Also many relatively small sinus fibres must be classified as type 3 (Fig 20 A) because the internodal bands continued right

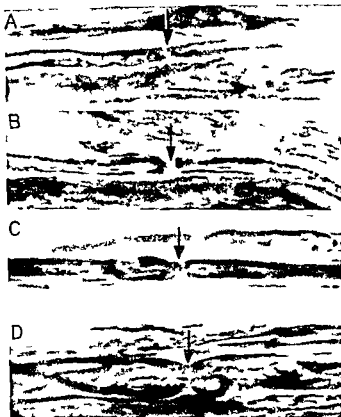


Fig 20 Light microphotographs of teased sinus nerve fibres fixed in glutaraldehyde and stained for $NADH_2$ tetrazolium reductase activity A—C from a 2 days old kitten D from a 35 days old kitten All fibres type 3 Marked internodal bands with accentuated staining at the paranodal region Arrows indicate node gaps All photos X 1380

up to the node gap forming paranodal stripes of accentuated staining (see Berthold and Skoglund 1967) Thus like the bronchial nerve fibres those of the sinus nerve show a generally more mature paranodal reductase pattern in relation to their size than the sural fibres at the newborn stage

At 35 and 45 days the adult pattern was reached in most of the fibres (Fig 20 D)

III *Respiratory responses to changes in oxygen content of the respired air in cats and rabbits*

Cats and rabbits showed essentially the same results

a) 21 % O_2—100 % O_2

1 Peripheral chemoreceptors intact

Anesthetized and decerebrate cats and rabbits of all ages from newborn to adults responded with a ventilatory diminution when the oxygen content of the

Fig 21 Diagrams showing changes in ventilation (Ve) and breathing frequency (F) in response to administration of pure oxygen as respired air in a 6 days old decerebrate kitten (A C) and an 8 weeks old Nembutal anesthetized (40 mg/kg b w) cat (B D) before (A B) and after (C D) denervation of the peripheral chemoreceptors

respired air was increased from 21 % (room air) to 100 % (Fig 21 A B) This indicates that the peripheral chemoreceptors are continually facilitating the respiratory center during air breathing already shortly after birth in these animals It must be pointed out that this response occurred also in animals where the common carotid arteries had been ligated and the vagi sectioned which confirms the finding of Chapter IV that the carotid chemoreceptors are still supplied with blood after ligation Moreover since ventilatory depression occurred also after denervation



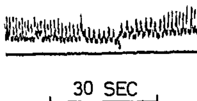


Fig Respiratory record (intraoesophageal pressure swings inspiration deflection upwards) showing the effect of administration of pure oxygen (arrow) after bilateral sinus nerve section (vagi intact) in a 6 days old decerebrate kitten The animal was breathing 10 % O in N_2 when pure oxygen was administred

of the carotid chemorepectors if the vagi were intact (Fig 22) also the arotic and/or pulmonary chemoreceptors are active in the newborn (Duke Green Heffron and Stubbens 1963)

In the animals below 3 weeks of age the ventilatory depression which occured with pure oxygen was often only transient after one or two minutes the ventilation began to increase and finally reached a value higher than when breathing room air (Fig 21 A) This cannot have been due only to a decrease in anesthetic depth since it occurred also in decerebrate animals and since administration of air again caused respiration to return to the original level (Fig 21 A) It must be stressed however that there were marked individual variations as regards this ventilatory improvement In the animals above 4 weeks on the other hand respiration regularly remained depressed during the 10 minutes test period (Fig 21 B)

2 Peripheral chemoreceptors denervated

After section of the vagus and sinus nerves which causes complete denervation of all known peripheral chemoreceptors administration of pure oxygen to animals les than 3 weeks old improved respiration considerably (Fig 21 C) Pure oxygen however did not affect animals above 4 weeks of age (Fig 21 D)

b) 21 % O —10 % O_2

1 Peripheral chemoreceptors intact

Both anesthetized and decerebrate cats and rabbits of all ages were found to respond with a respiratory augmentation when the oxygen content of the respired air was reduced from 21 % (room air) to 10 % However in the animals below 35 days of age this increase was only transient and during the 10—20 minutes test period respiration subsequently decreased progressively (Fig 23 A) The respiratory diminution was most marked in animals below 10—14 days of age In a few animals about one week old respiration failed completely after some 10—15 minutes exposure to 10 % O_2 or became gasp like (Fig 24) In contrast the animals above 8 weeks of age regularly maintained hyperventilation (Fig 23 B)

To ensure that the ventilatory depression in the youngest animals with intact peripheral chemoreceptors was not accompanied by a cessation of the receptor discharge recordings were made from a thin filament of the right sinus nerve while the other one and the vagi were intact As can be seen in Fig 25 the discharge only increased when the ventilation ceased This means that the center must have become unresponsive to the peripheral input In response to one or a few artificial inflations of pure oxygen the chemoreceptor discharge disappeared completely (Fig 25)

2 Peripheral chemoreceptors denervated

In the animals less than 3 weeks either anesthetized or decerebrated the respiration already greatly depressed by the denervation (cf Chapter IV) rapidly decreased further and often apnoea ensued within a few minutes (Fig

Fig 23 Diagrams showing changes in ventilation and breathing frequency in response to administration of 10 % O_2 in N_2 as respired air in a 5 days old Nembutal anesthetized (30 mg/kg b w) kitten (A, C) and an 8 weeks old Nembutal anesthetized (40 mg/kg b w) cat (B D) before (A, B) and after (C D) denervation of the peripheral chemoreceptors

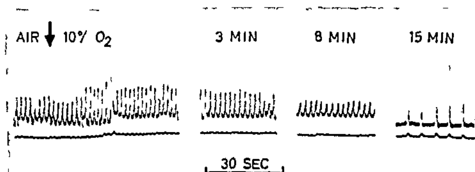


Fig 24 Respiratory records (as in Fig 22) from a 9 days old Nembutal anesthetized (35 mg/kg b w) kitten, showing the effect of hypoxia Administring 10 % O_2 in N_2 (arrow) caused a transient increase during the first minutes and a secondary decrease of respiration After 15 min a gasp-like breathing pattern ensued

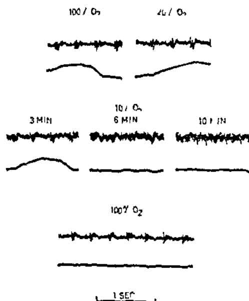


Fig 25 Records showing the discharge in a thin filament of the right sinus nerve (upper beam) and respiration (lower beam as in Fig 7) in a 7 days old Nembutal anesthetized (30 mg/kg) kitten breathing pure oxygen room air and 10 % O in N (the left sinus nerve and both vagi intact) After 6 min of hypoxia the respiratory activity ceased in spite of an increased firing in the sinus nerve A few artificial inflations of pure oxygen reduced this discharge completely (lowermost record)

23 C) However sometimes a gasp like breathing pattern occurred which was maintained for a considerable period of time

The effect of administration of 10 % O to the animals above 4 weeks of age after chemorepector denervation varied with the anesthetic depth Deeply anesthetized animals were rapidly depressed and apnoea ensued within a few minutes (Fig 23 D) In more lightly anesthetized animals on the other hand respiration continued unaltered (cf Schwieler 1967)

IV *Changes in arterial pCO_2 and pH during hypoxia in newborn kittens*

A decrease in ventilation should if the CO_2 production is constant result in an accumulation of CO This was tested by continuously recording the arterial pH and pCO and ventilation during exposure to 10 % O_2 in 3 kittens about 1 week old A typical experiment is illustrated in Fig 26 When 10 % O_2 was administred there was a characteristic biphasic ventilatory response In spite of the decrease in ventilation however pCO_2 continued to fall and pH to rise When 10 % O was replaced by pure oxygen pCO_2 again increased as did ventilation (after a short initial decrease) and the pH fell

Discussion

The results show clearly that the peripheral chemoreceptors are functional in newborn kittens and rabbits In both these species the receptors apparently facilitate the respiratory center continuously during air breathing since administration of 100 O caused a ventilatory diminution before but not after their

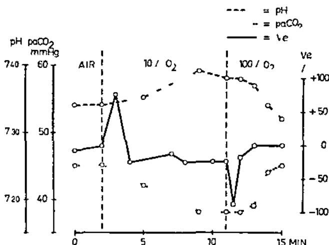


Fig .6 Diagram showing the changes in ventilation arterial pH and pCO in response to administration of 10 % O in N₂ in a 7 days old Nembutal anesthetized (30 mg/kg) kitten

denervation Since an increase in O of the respired air also depressed ventilation after sinus nerve section when the vagi were intact this is evidence that the aortic and/or pulmonary chemoreceptors are functional at birth Direct recordings from sinus nerve affe ents (carotid chemoreceptors) in newborn kitten showed that at lea t some receptors were continually active during air breathing They decreased their frequency or ceased discharging in response to 100 % O_2 while there was an increase in frequency and recruitment of new units in response to 10 % O However in spite of the ability of the receptors to fire continually the ventilatory response to hypoxia was biphasic in the animals below 4 weeks of age This could not be attributed to a decrease in chemoreceptor discharge since these fired continually and even increased their discharge rate when ventilation decreased This discharge thus seems to be less effective during hypoxia Simultaneously with the ventilatory diminution the arterial pCO decreased indicating a simultaneous decrease in the oxidative metabolism

Since different receptors may give similar spike amplitude a continual firing pattern might arise from the contributions of two or more receptors firing intermittently Recordings were however also made from a few single units which in fact proved to fire continually No detailed frequency analysis was made From the records obtained however the frequency ranges seem to be rather small (less than 10/sec in air and between 10—30/sec in 10 % O) and it could thus be argued that this is the reason why these fibres are able to fire continually However Ekholm (1967) found that sural nerve fibres in the newborn are unable to continue firing with a frequency of 25/sec for more than a few seconds when stimulated electrically Thus it seems likely that the carotid chemoreceptor affer

ents are more mature functionally at birth than the sural nerve afferents

From a recent report by Biscoe Sampson and Purves (1967) is appears that the frequency ranges for carotid chemoreceptor afferents of adult cats are of the same order as those obtained from the newborn kittens in the present work This further supports the assumption the these afferents are nearly mature at birth However since in the present work the arterial pO_2 was not recorded at the same time as the electrical activity of the chemoreceptor afferents no further comparisons can be made

The next step then was to study in the sinus nerve fibres those morphological characteristics which it has been suggested are correlated with the functional maturity viz the fibre diameter and paranodal $NADH_2$ reductase pattern (see Romero and Skoglund 1965 Berthold and Skoglund 1967) and to compare the results with those obtained from the sural and bronchial nerves of the same animals (Chapter V) As regards the fibre composition the sinus nerve fibres were generally smaller than those of the sural and bronchial nerves at all developmental stages On the other hand as regards the maturation of the node paranode region (see p 53 and Berthold and Skoglund 1967) the largest sinus nerve fibres were like those of the bronchial nerve found to be more mature than sural fibres of similar or even larger size Thus again there appears to be a correlation between the mature paranodal reductase pattern and the ability to fire continually Since the method of recording is likely to give rise to a biased selection of the biggest fibres the records in the present work are probably from the most mature fibres in the sinus nerve as were those in the work of Ekholm (1967) on the sural nerve

It could be argued that the largest fibres of the sinus nerve are pressoreceptor afferents since in the adult the spikes recorded from these afferents are generally larger than the chemoreceptor spikes (see Heymans and Neil 1958) However in the newborn stage the differences in size between the different afferents are less as seen from the calibre spectrum Moreover some of the spikes filling in the pauses between the pressoreceptor dischargs are seen to be of the same order of size as the latter (Fig 17 p 51 and Fig 25 p 58)

From these results and those of the preceeding Chapter with regard to the comparisons between the functional and morphological properties of the bronchial sinus and sural nerves during postnatal development it may be concluded that in spite of the fact that the bronchial and sinus nerves are composed of fibres of similar or even smaller size than those in the sural nerve at birth they appear to be more mature both functionally and with regard to the paranodal reducta e pattern Thus there is experimental evidence as suggested by Berthold and Skoglund (1967) that the maturity of the node paranode region is a better correlated to functional maturity than fibre size (see also General Discussion) The finding that the sinus and bronchial nerves are functionally ahead of the sural nerve gives further support to the generally held view of a cranio-caudal

development of the central nervous system (Kingsbury 1932)

The initial response of newborn and adult to 10 % O_2 is the same hyper ventilation and reduced arterial pCO_2 (see Results section IV and Bjurstedt 1946) In spite however of the fact that the peripheral chemoreceptors are relatively mature in the newborn ventilation subsequently began to decrease progressively in all animals below 4 weeks old at the environmental temperature used here This is in accordance with earlier findings (Adamsons 1959 Dawes and Mott 1959) There is ample evidence that the pulmonary compliance decreases during hypoxia in newborn animals (Purves 1966 b) Thus the pressure amplitude will give an overestimate of the inspired volumes and the real ventilatory decrease will be even larger than that calculated (ee Chapter IV p 31) Simultaneously with this ventilatory depression the arterial pCO de creased This probably reflects a decrease of endogenous CO_2 production and thus of aerobic metabolism which is also in accord with earlier findings (Adam sons 1959 Dawes and Mott 1959) This decrease in pCO_2 might actually partly explain the ventilatory diminution since the stimulation of the central chemo receptors (Chapter VIII) thereby decreases However, the decrease in pCO_2 cannot be the whole explanation for the ventilatory diminution since in some cases in the present work respiration ceased completely Moreover Brady and Ceruti (1966) reported that adding CO_2 to the respired air did not prevent the ventilatory depression in newborn infants

Since there is a slight postnatal increase in the total number of myelinated fibres and type 3 fibres in the sinus nerve the ventilatory response to 10 % O_2 in the newborn may be quantitatively less resulting in a greater relative decrease in the oxygen saturation of the blood and hence a more intense central dep es sion However lightly anesthetized or decerebrate animals above 8 weeks were not even depressed by 10 % O_2 after denervation of the peripheral chemore ceptors In decerebrate newborn animals on the other hand with intact chemo receptors ventilation always decreased progressively after an initial augmentation Thus there is evidence that the newborn is more sus eptible to a decrease in oxygen supply than the adult with regard to respiration Since the chemoreceptor discharge increased progressively when ventilation was depressed this discharge thus appears to become less effective during hypoxia Firstly the central affe ent terminals might become blocked due to hyperpolarization when firing at high fre quencies which apparently is the case in the spinal cord with the immature muscle afferents (Skoglund 1960 e) Secondly hypoxia might change the central threshold for the receptor discharge Reynolds and Mackie (1961) found evidence that the threshold for the chemoreceptor discharge decreases if the oxygen supply is increased and Godfrey (1966) in newborn rabbits found that the pulmonary tension receptor discharge does not affect the respiratory neurons during asphyxia The possible relationship between a change in oxidative metabolism and central

threshold will be further taken up in the General Discussion

The possibility that the central threshold may change when the oxygen supply is changed is further supported by the fact that ventilation often increased (after an initial diminution) when pure oxygen was administred Cross and co-workers (see Cross 1964) Miller (1954) and Girard Lacaisse and Dejours (1960) have found that hyperoxia augments ventilation also in the newborn infants Cross (1964) has discussed different theories to explain this finding one of them being that the high oxygen has an irritant effect on the lungs This cannot be the whole explanation however since it was found in the present work, that 100 % O_2 also improved ventilation after vagotomy Although this increase in ventilation might partly be due to respiration being already severely depressed after the nerve section (cf Chapter IV) and the animal consequently hypoxic the findings support the view that the high oxygen pressure in the medulla causes increased responsiveness of the medulla (Cross 1964)

In the adult there is a direct relationship between oxygen consumption and ventilation (see Gray 1950) and by analogy it has been suggested (see Brady and Ceruti 1966 Purves 1966 b) that the secondary ventilatory depression during hypoxia in the newborn is a consequence of the decrease of oxygen consumption However there is little real evidence for this view because the parallelism between oxygen consumption and ventilation in the adult was found in tests during exercise and Matell (1963) showed that such ventilatory changes could also be explained by changes in the arterial pH

Hill (1959) reported that small adult animals (guinea pigs) like newborn animals of similar size (kittens) but in contrast to larger adult ones (dogs) reduced their oxygen consumption when exposed to 10 % O_2 at an environmental temperature below neutral However she did not compare possible changes in ventilation and it remains to find out whether the reaction to hypoxia of an adult guinea pig is the same as that of a newborn kitten in this respect

Finally it may be noted that the fibre composition of the sinus nerve in the adult showed slightly smaller fibres than that obtained by Eyzaguirre and Uchizono (1961) However this may be explanied by the different fixation methods used and that they measured the diameters of the circumscribed circles (cf Chapter V)

Summary

1) Recordings from thin filaments of the sinus nerve and from single carotid chemoreceptor afferents were made in newborn and young kittens breathing air 100 % O_2 and 10 % O_2 in N_2 A tonic receptor discharge present when the animals breathed air decreased on 100 % O_2 and increased on 10 % O_2 The frequency in a few single receptors in newborn kittens was found to be less than 10/sec in air and between 10 and 30 sec in 10 % O_2

2) The calibre spectrum and paranodal $NADH_2$ tetrazolium reductase activity of single nerve fibres were analyzed in the sinus nerve during postnatal develop ment and the results compared with the corresponding findings from the bronchial and sural nerves in Chapter V The sizes of the fibres comprising the peak fre quencies as well as the biggest fibres were found to be smaller in the sinus than in the bronchial and sural nerves at birth Postnatally the sinus nerve fibres were found to grow much less than those of the bronchial and sural nerves Like bronchial nerve fibres however the sinus fibres showed a more mature paranodal reductase pattern than sural fibres of the same or even larger size

3) The relation between functional and morphological maturation of the nerve fibres during postnatal development is discussed

4) The ventilatory responses to changes in oxygen content of the respired air before and after denervation of the peripheral chemoreceptors were studied in cats and rabbits during postnatal development When the chemoreceptors were intact an increase of the oxygen content gave an initial ventilatory diminution whereupon respiration progressively increased in the animals below 4 weeks of age Decreasing the oxygen content to 10 % gave a transient ventilatory increase in animals below 4 weeks of age followed by a ventilatory depression while animals above 8 weeks remained hyperventilating After chemoreceptor denerva tion pure oxygen produced a ventilatory increase in the youngest animals and no change in the older ones 10 % O_2 gave a ventilatory depression in all animals below 4 weeks of age and in fully anesthetized animals above 8 weeks of age but no change in lightly anesthetized older animals

5) The arterial pCO_2 and pH were recorded continually in a few one week old kittens during hypoxia Simultaneously with the ventilatory depression the pCO_2 decreased and the pH rose This may be explained by a decrease in oxidative metabolism

6) The present results are discussed and it is concluded that part of the explanation for the decrease in ventilation in response to hypoxia is that the central drive decreases because of reduced central stimulation It is also possible that the center becomes unresponsive to the receptor discharge

solution or paraffin oil at 37°C The dural sac was not opened until just before sectioning the roots Respiration was recorded as the intraoesophageal pressure variations (see page 18) Tracheal occlusion was performed simply by placing a finger on the cannula opening between the end of one expiration and the end of the next expiration i e through one breathing cycle

Calibre spectra

The fibre calibre analysis was made on the right 3rd and 6th thoracic dorsal and ventral roots and intercostal nerves and on the right phrenic nerve taken from five newborn two 7 days two 10 days old kittens one 25 days old kitten and one adult cat The nerves were removed from the living animal under Nembutal anesthesia fixed in 0 5 % osmic acid for 24 hours and dehydrated in alcohols and embedded in paraffin Sections were cut at 3—5 μ thickness Selected sections were then photographed on roll film directly in the microscope and the films were enlarged to an overall linear magnification of 1 000 (see Romero and Skoglund 1965) The nerve fibres were measured with a particle size analyzing machine (Zeiss TGZ 3) according to the method of Romero and Skoglund 1965)

The present method does not give the same good quality of the preparations as the method used in Chapter V and VI However since in the present investigation it was desired to compare the results of the fibre calibre analysis with those of the lumbar roots obtained by Skoglund and Romero (1965) the method used by them was followed

The quality of the preparations varied extremely and some which were not satisfactory for analysis were discarded and hence all the nerves examined are not represented in the results Further the quality between different fascicles within the same nerve or root often varied and some of the fascicles could not be measured In each case all the fibres of qualitatively satisfactory fascicles were measured The actual total number of measured fibres in corresponding nerves from different animals thus varies considerably and is not an index of the total number of fibres in any case

Histochemistry

Parts of the 3rd 6th and 10th external intercostal muscles and of the diaphragm were excised from lightly Nembutal anesthetized cats and kittens (4 kittens aged 1—2 days 3 kittens 6—10 days 3 kittens 21—35 days and 2 adult cats) The muscle pieces were immediately fixed in liquid pentane previously cooled with liquid nitrogen The specimens were then either stored in a refrigerator at —70°C or directly sectioned in a cryostat into 10—15 μ thick sections Succinic dehydrogenase was then tested for by the method of Nachlas Tsou De Souza Cheng and Seligman (1957)

I *The effect of different nerve sections and of increased respiratory load on the inspiratory intercostal muscle activity*

The findings in cats and rabbits were in general the same

a) The effect of section of the vagus nerves

In cats and rabbits of all ages section of the vagus nerves caused a considerable increase in inspiratory intercostal muscle activity (EMG) the immediate effect was a prolongation of the subsequent inspiratory phases (Fig 27) This indicates that at all ages the vagal afferents exert an inhibitory effect on the inspiratory activity of the intercostal muscles After a few minutes however respiration became more varied in the animals below 2—3 weeks while a prolonged inspiratory phase lasted for up to an hour in most animals there was a progressive shortening of the inspirations in some animals and in others the breathing became periodic or gasp like (see Chapter IV) Accordingly also the activity of the intercostal muscles varied after vagotomy

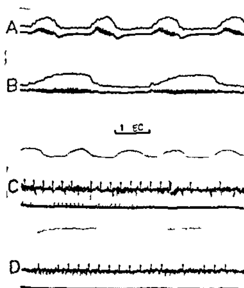


Fig 7 Records of respiration (upper beams intraoesophageal pressure swings inspiration deflection upwards) and external intercostal muscle EMG (lower beams) from a 35 days old decerebrate kitten (A B) and a 1 day old decerebrate rabbit (C D) before (A C) and after (B D) vagotomy *The EMG records in C and D show* interference from ECG Time 100 msec

b) The effect of section of the thoracic dorsal roots

The EMG was recorded from the 4th or 5th intercostal muscle and 2 to 4 dorsal roots above and below this segment were also sectioned bilaterally There was a pronounced decrease in the intercostal muscle activity in kittens above about 10 days old and rabbits above 14 days old (Fig 28 A) as in adult animals (Euler and Fritts 1963 Sant Ambrogio and Widdicombe 1965) In the newborn on the other hand no such changes occurred after rhizotomy (Fig 28 B) Since

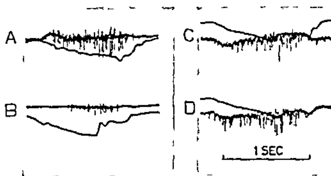


Fig 29 Records of respiration (intraoesophageal pressure swings inspiration deflection downwards) and external intercostal muscle EMG from a 28 days old (A B) and a 1 day old (C D) decerebrate vagotomized kitten before (A C) and after (B D) section of 7 pairs of thoracic dorsal roots The rhizotomy caused a considerable decrease in EMG activity in the older but not in the yonger animal

decerebrate animals were used this absence of effect cannot be explained by a greater sensitivity of the newborn to the anesthetic with a consequent masking of afferent input The section of the roots had to be made with greatest caution in order not to affect the ventral roots In a few cases a slight decrease in EMG activity was also observed in newborn animals after rhizotomy In these cases however it was not possible to exclude that the ventral roots had been damaged A negative result in fact was taken as evidence that the ventral roots had not been affected It was always checked afterwards that altogether at least 5 pairs of dorsal roots had been completely cut

c) The effect of tracheal occlusion

By occluding the tracheal cannula between the end of one expiration and the end of the next expiration the expansion of the thoracic cavity and hence of the lungs is severely limited during inspiration Thus the inspiratory intercostal muscles are subjected to increased load since there is resistance to their shortening (Corda Eklund and Euler 1965) The effect of such an occlusion was only studied if respiration was previously constant in amplitude in order not to interfere with the spontaneous variations which were sometimes seen after vagotomy

When the innervation was intact cats and rabbits of all ages responded to tracheal occlusion with an increase in the activity of the inspiratory intercostal muscles as is illustrated in Fig 29 A After vagotomy tracheal occlusion only caused an increased inspiratory intercostal muscle activity in cats and kittens above 10—14 days and in rabbits above 15—20 days of age (Fig 29 B) In younger animals the EMG did not usually change (Fig 29 C) occasionally there was a slight decrease of activity manifested by a shortened period of activity (Fig 29 C and Fig 0 A) When vagotomy was followed by rhizotomy tracheal occlusion did not affect the EMG activity (Fig 30 B) at any age which indicates

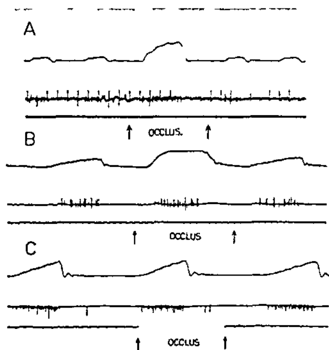


Fig 29 Records of respiration (upper beams as in Fig 27) and internal intercostal muscle (intercartilaginous part) EMG (lower beams) from three decerebrate rabbits aged 1 day (A C) and 28 days (B) old A intact innervation B C vagi sectioned Tracheal occlusion (between arrows) caused an increase in EMG activity in A and B but not in C The EMG record in A show interference from ECG Time 100 msec

that the changes after vagotomy alone were mediated by spinal reflexes

Kittens and rabbits above some 10 and 15 days of age respectively thus seem to have acquired an intercostal load compensating mechanism mediated via the thoracic dorsal roots and similar to that of the adult animals (Campbell and Howell 1962 Corda Eklund and Euler 1965 Sant Ambrogio and Widdicombe 1965) Kittens and rabbits below these ages appear to lack such a control mechanism Since in the adult this has been shown to be an autogenetic reflex equivalent to the stretch reflex (Corda Eklund and Euler 1965) the absence of this reflex in the newborn indicates that their intercostal muscle spindles do not function properly (see Discussion)

It may be pointed out that in the newborn the change in the inspiratory intercostal muscle activity in response to tracheal occlusion was very similar to the initial increase in activity caused by vagotomy (Fig 31) Thus in spite of the fact that the newly born animals lack a spinal control mechanism which increases

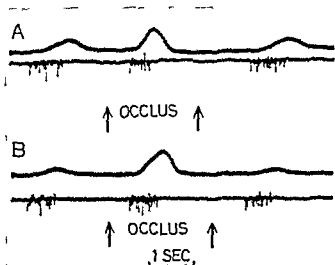


Fig 30 Records of respiration (upper beams as in Fig. 27) and external intercostal muscle EMG (lower beams) from a 2 days old decerebrate vagotomized kitten before (A) and after (B) section of 6 pairs of thoracic dorsal roots Tracheal occlusion (between arrows) caused a decrease in the EMG activity before but not after the rhizotomy

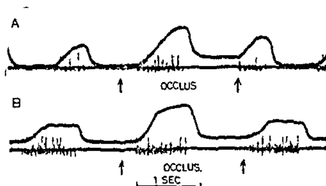


Fig 31 Records of respiration (upper beams as in Fig 27) and internal intercostal muscle (intercartilaginous part) EMG (lower beams) from a 10 days old decerebrate rabbit (A) before and (B) after vagotomy Tracheal occlusion (between arrows) caused an increase in EMG activity before vagotomy similar to that caused by the vagotomy

the intercostal activity in response to increased inspiratory load the release of inhibition from the pulmonary tension receptors which is a consequence of the tracheal occlusion actually serves a similar purpose It cannot be excluded however that tracheal occlusion also activated the pulmonary deflation receptors"

(Paintal 1955, 1957) and consequently facilitated the inspiratory motoneurons as suggested by Euler and Fritts (1963)

II *Calibre spectra of thoracic spinal roots and intercostal and phrenic nerves in the cat*

The results of the measurements are presented as calibre spectra in tables VI—XII and as histograms in Fig 32

a) The growth of the 3rd and 6th thoracic spinal roots

At birth most of the myelinated nerve fibres are between 2 μ and 3 5 μ in diameter while the biggest fibres have reached some 4—5 μ The *dorsal root* fibre diameters then spread out progressively over a broad range and at 25 days most of the diameters are relatively evenly distributed between 2 μ and 6 μ with a low peak at about 4 μ In the adult stage the biggest fibres are some 16 μ to 18 μ in diameter and there is a relative increase in fibres between 2 5 μ and 5 μ This developmental pattern is in general similar to that of the lumbar dorsal roots in the cat as described by Skoglund and Romero (1965)

The *ventral root* fibre diameters spread out progressively over a similar range as the dorsal ones at the corresponding ages However already at 25 days these clearly show two different peaks one at about 2 μ and the other at about 4—6 μ In the adult these two peaks are still present but here shifted to about 3 μ and some 10—14 μ respectively

A comparison with the lumbar ventral roots of the same ages (Skoglund and Romero 1965) reveals two important differences Firstly the peak of fibres of about 2 μ was not found in the lumbar roots at 25 days of age although such a peak supposed to comprise the gamma fibres was present at 37 days Secondly when present this peak was relatively smaller in the lumbar than in the thoracic ventral roots This was so also in the adult stage The same general differences between the thoracic and lumbar roots were also found in man by Rexed (1944)

When the ventral and dorsal roots are compared there is the impression that the latter lag somewhat behind in development This was supported by the finding that in kittens 1 7 and 10 days of age a relatively greater number of fibres were above 3 42 microns in the ventral than in the dorsal roots (Table XIII) The difference between the dorsal and ventral roots was significant at the 5 % level with regard to the 3rd segment and at the 1 % level with regard to the 6th segment when all animals were included and tested by a non parametric method (see Siegel 1956 pages 68—75) This method has been used since it does not require that all observations are drawn from the same age group and it was also assumed that all missing values (i e those nerve sections unsuitable for measurement see page 66 are random This difference is in general the same as that found in the lumbar region in the cat (Skoglund and Romero 1965) and in all spinal segments

Table VI The calibre spectrum of Th 3 V

Age in days	Animal nr	1 21 1 76	1 76 2 3	2 3 2 87	2 87 3 42	3 42 3 97	3 97 4 5	4 5 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 39 8 9
1	KT 15	8	105	238	210	59	6								
1	KT 21	8	60	178	178	66	11								
7	KT 22	3	13	5	106	97	31	12	1	1					
7	KT 23	29	37	96	207	239	115	11	6	5					
10	KT 14	6	47	55	67	91	119	91	16	18	8				
10	KT 24		32	88	113	138	107	50	8	2					
25	KT 17	23	167	1 8	34	76	54	61	83	52	41	11	10	7	1
adult	KT 13	2	18	119	16	86	29	20	16	9	8	4	10	15	15

Table VII The calibre spectrum of Th 6 V

Age in days	Animal nr	1 21 1 76	1 76 2 32	2 32 2 87	2 87 3 4	3 4 3 97	3 97 4 52	4 52 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 39 8 94
1	KT 15	1	23	107	142	108	38	3							
1	KT 19		41	151	170	82	39	7	2						
1	KT 20		51	40	224	170	31	4	2						
1	KT 21	3	16	104	152	113	36	1							
2	KT 16		74	261	35	95	13	1							
7	KT 23	24	91	186	251	203	5	29	1	-					
10	KT 14		--	76	1 4	129	100	52	32	7	2				
10	KT 24		13	69	99	153	170	170	160	19	5	1			
25	KT 17	6	144	103	45	46	103	96	108	73	30	11	-		
adult	KT 13	1	18	35	55	32	25	15	7	2	-	3	2	4	18

Table VIII The calibre spectrum of Th 3 D

Age in days	Animal nr	1 21 1 76	1 76 2 32	2 32 2 87	2 87 3 4	3 42 3 97	3 97 4 5	4 5 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 39 8 94
1	KT 15	2	36	129	122	14	1								
1	KT 19	10	99	155	97	49									
1	KT 20	0	139	117	29	3									
1	KT 21	5	211	2 6	146	16	1								
2	KT 16	8	26	45	102	20	1								
7	KT 22		137	31	213	25	3								
7	KT 23		3	177	2 4	13	5	6	2						
10	KT 14	10	76	183	135	105	48	0	5						
10	KT 24	8	65	160	214	179	114	36	12	1					
25	KT 17	6	54	138	115	1 5	117	71	62	37	13	1	4		
adult	KT 13	2	16	7	95	83	81	57	45	50	35	50	39	33	33

0 1.	11 1. 11 70	11 70 1 5	1 .. 1. 80	1 80 13 36	13 36 13 91	13 91 14 4.	14 46 15 01	15 01 1 56	1 56 16 1.	16 12 16 67	16 67 17 2.	17 2 1. 77	Numb r of fibres measured
													6 6
													451
													316
													803
													551
													538
													708
3	38	18	.8	25	17	9	8	4	3	2	2	1	764

C0 15	11 15 11 70	11 0- 12 25	12 .5 1. 80	12 80- 13 36	13 36 13 91	13 91 14 45	14 45 15 01	15 01 15 56	15 66 16 1.	16 12 16 67	16 67 17 2.	17 . 17 77	Number of fibres m asured
													422
													507
													675
													428
													679
													842
													545
													711
													787
24	16	11	12	8	12	4	5		1	1	1	1	310

60 1 15	11 15 11 70	11 70- 1 25	1 .5 1 80	1 80 13 36	13 6 13 91	1 91 14 46	14 4 15 01	15 01 15 56	15 56 16 12	16 12 16 67	16 67 17 ..	17 2 17 77	Numb r of fibres measured
													304
													410
													308
													63
													679
													71.
													618
													582
													789
													754
21	11	11	8		3	1		1					845

Table IX The calibre spectrum of Th 6 D

Age in days	Animal nr	1 21 1 76	1 76 2 32	2 32 2 87	2 87 3 42	3 42 3 97	3 97 4 52	4 52 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 [illegible] 8 [illegible]
1	KT 15	4	62	176	56	2									
1	KT 19	3	47	180	166	37									
1	KT 20	21	168	109	30										
1	KT 21	24	208	144	13										
2	KT 16	67	291	243	108	7									
7	KT 22	32	138	99	174	70	7								
7	KT 23	7	91	182	130	46	16	2							
10	KT 14	14	117	220	211	137	55	1							
10	KT 24	7	65	135	185	165	67	19	1	2					
25	KT 17	4	11	83	108	145	136	170	109	61	37	29	16	[illegible]	
adult	KT 13	2	8	29	67	50	39	47	36	27	31	29	23	[illegible]	[illegible]

Table X The calibre spectrum of Th 3 I

Age in days	Animal nr	1 21 1 76	1 76 2 3	2 32 2 87	2 87 3 4	3 4 3 97	3 97 4 52	4 52 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 3 8
1	KT 19	5	78	150	111	36	11								
1	KT 20	6	80	146	139	90	20	2							
2	KT 16	1	37	170	101	65	14								
7	KT 22	7	74	84	97	90	56	19	1						
10	KT 14	15	31	122	127	114	93	92	22						
25	KT 17		13	31	45	43	56	38	39	3	46	45	24	20	
adult	KT 13				6	8	8	14	19	3	5	12	11	11	

Table XI The calibre spectrum of Th 6 I

Age in days	Animal nr	1 21 1 76	1 76 2 3	2 32 2 87	2 87 3 42	3 42 3 97	3 97 4 52	4 52 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 3[illegible] 8 [illegible]
1	KT 20	73	135	114	137	87	25	6							
2	KT 16	61	139	95	104	80	39	12	1	1					
7	KT 22	15	119	131	127	99	80	25	3						
10	KT 14	22	97	11	101	70	80	52	27	12	5				
25	KT 17	18	11	78	67	72	54	63	56	57	45	31	2	6	[illegible]
adult	KT 41				6	7	16	1	34	17	12	14	[illegible]	15	1

60 15	11 15 11 70	11 70- 1 2	12 5 1 80	1 80 13 6	13 6 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 1	16 1 16 67	16 67 17	17 2 17 77	Number of fibres measured
													50
													439
													337
													389
													719
													720
													496
													758
													646
													917
7	21	16	27	[illegible]1	17	8	7	6	7		3	1	643

60 15	11 15 11 70	11 70- 1 5	1 5 1 80	1 80- 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 1	16 1 16 67	16 67 17 22	17 17 77	Number of f bres measured
													391
													484
													343
													434
													618
													438
11	14	11	9	7	5	1	1						196

0 60 11 15	11 15 11 70	11 70- 12 25	1 5 1 80	1 80- 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 12	16 12 16 67	16 67 17 22	17 17 77	Number of fibres measured
													577
													532
													594
													591
													616
11	14	27	18	3	26	26	29	15	22	16	9	1	46[illegible]

Table XII The calibre spectrum of n phrenicus

Age in days	Animal nr	1 21 1 76	1 76 2 3	2 32 2 87	2 87 3 42	3 4 3 97	3 97 4 5	4 5 5 08	5 08 5 63	5 63 6 18	6 18 6 73	6 73 7 28	7 28 7 84	7 84 8 39	8 39 8 91	8 9
1	KT 19	31	170	204	75	28	13									
1	KT 20	12	119	209	180	97	35									
2	KT 16	8	68	138	101	60	20	4								
7	KT 22	?	41	156	166	145	77	28	3	2						
7	KT 23		29	178	187	139	112	13	1							
10	KT 14	2	16	40	47	93	147	151	95	31	13					
25	KT 17	4	8	26	42	67	87	105	110	57	35	10	1			
adult	KT 13			3	4	11	1	13	15	1	4	9	9	10	12	1

Table XIII Percent of fibres above 3 42 microns in the 3rd and 6th thoracic dorsal and ventral roots and in the phrenic nerves in 9 kittens aged 1 to 10 days

	1—2 days					7 days		10 days
Animal nr / Nerve	KT 15	KT 19	KT 0	KT 1	KT 16	KT	KT 23	KT 14
Th 3 D	5	10	1	3	3	4	30	31
Th 6 D	1	7	0	0	1	11	13	26
Th 3 V	11	—	—	17	—	—	56	68
Th 6 V	37	28	23	36	16	45	32	50
Phren	—	8	20	—	21	41	44	84

in man (Rexed 1944) and may be explained by the earlier myelinization of the ventral roots (Ambronn and Held 1896)

b) The growth of the 3rd and 6th intercostal nerves

The intercostal nerves in the cat after leaving the dorsal spinal rami divide into single internal and external branches the former innervating the internal intercostal as well as the abdominal muscles and the skin of the thorax and abdomen and the latter innervating only the external intercostal muscle (Eccles Sears and Sealy 1962 Sears 1964) Thus the internal branch is a mixed nerve whereas the external branch is a pure muscle nerve In the present work the external branches of the 3rd and 6th intercostal nerves were used for analysis

The fibre diameters of the external intercostal nerves also spread out progressively over the same ranges as the spinal roots at the corresponding ages At 25

10 60 11 15	11 15 11 70	11 70 1? .5	12 .5 1 0	1 80 13 36	13 36 13 91	13 91 14 4	14 46 15 01	15 01 15 56	15 56 16 1	16 1 16 67	16 67 17 2.	17 17 77	Number of fibres measured
													521
													660
													399
													620
													409
													635
													55.
17	26	32	13	19	13	9	1	1					305

days of age however an important difference between the ventral roots and intercostal nerves is the lack of a pronounced peak at about 2 μ in the latter This may be because the thoracic ventral roots also contain preganglionic sympathetic fibres whereas the intercostal nerves do not This might also very well be the reason why the relative size of the peak comprising the smallest fibres is larger in the ventral roots than in the intercostal nerves in the adult stage

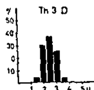
c) The growth of the phrenic nerves

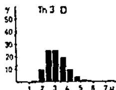
The growth of this nerve very closely follows that of the ventral roots during the first 10 days and thus precedes the dorsal roots in development (Table XIII) Whereas the difference between the phrenic nerves and the 6th thoracic dorsal roots was significant at the 5 % level that between the phrenic and the 3rd thoracic dorsal root was not significant using the non parametric method (page 71) At 25 days old and in the adult however the phrenic nerve showed a clear dominance of larger fibres which can be explained by the very small number of afferent fibres it contains (some 10 % Hinsey Hare and Phillips 1939)

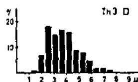
III *Succinic dehydrogenase in the diaphragm and intercostal muscles*

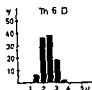
At the newborn stage all muscle fibres of the intercostal muscles and the diaphragm stain only weakly for succinic dehydrogenase although a slight difference between somewhat darker and lighter fibres is visible (Fig 33 A) This difference then increases and is clearly seen at the 6 days stage (Fig 33 B) Already at the 25 days stage three types of fibres can be indentified (Fig 33 C) as in the adult stage (Fig 33 D) (a) darkly stained (b) lightly stained and (c) intermediate With the method used here no clear differences between different intercostal muscles or between these and the diaphragm could be seen in preparations from the same developmental stages

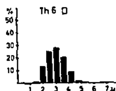
1 2 DAYS | 10 DAYS | 5 DAYS

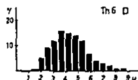


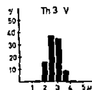


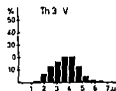


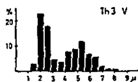


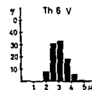


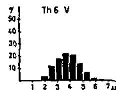


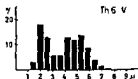




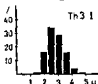


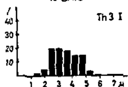


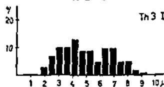


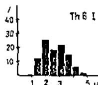


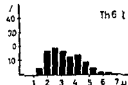
1 2 DAYS | 10 DAYS | 25 DAYS

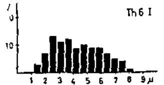


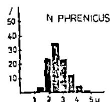


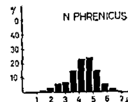


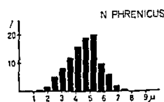


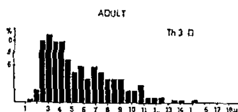


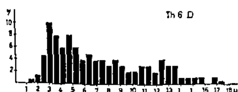












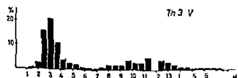








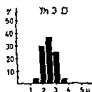


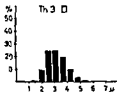
Fig 32 Histograms of calibre spectra of thoracic spinal roots and intercostal and phrenic nerves from cats of different ages indicated in the figure The histograms from the 1—? and 10 days old animals are composed of the calibre spectra from all animals at each respective stage re presented in the Tables Note different scales on the ordinates

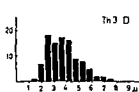
1 2 DAYS

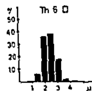
10 DAYS

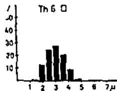
5 DAYS

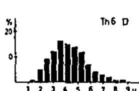


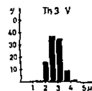


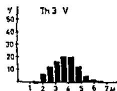


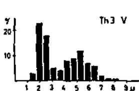


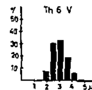


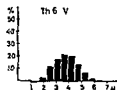


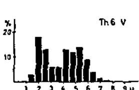








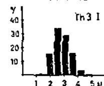


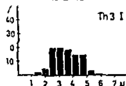


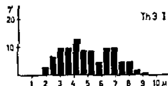
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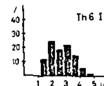
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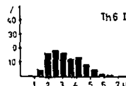
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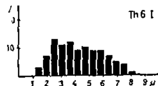


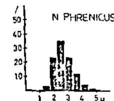


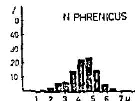


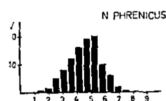












This may very well have been mediated by the Golgi tendon organs but requires further study

What then is the reason for the absence of a tonic stretch reflex in the intercostal musculature of the newborn animal? Skoglund (1960 a c) found that such a reflex was also lacking in the distal hindlimb mus les of the newborn kitten By recording from lumbar dorsal root afferents he showed that although the muscle spindles responded with a discharge when the muscle was stretched the discharge was weak had a low frequency and was phasic in character Moreover in the neurogram of the muscle nerve stimulated from the ventral root a gamma elevation was not clearly visible until some time after birth (Skoglund 1960 b) Finally the muscle spindles could not be influenced by gamma activation All these properties were found to develop gradually and to be present earlier in the proximal than in the distal muscles Skoglund concluded that the absence of a tonic stretch reflex could be attributed to the absence of gamma control of the muscle spindles By analogy the same conclusion might be drawn from the present work and information about the activity of single spindle afferents would be of great value Such an approach has also been tried but the difficulties of recording from the thoracic dorsal roots have not yet been overcome due to their extreme shortness in the newborn However the results of the morpological investigation give further support to the indirectly reached conclusion (see below)

It may be pointed out that the EMG recordings were made either dorsally from the external intercostal muscles or ventrally from the intercartilaginous part of the internal intercostal muscles Both these parts of the intercostal muscles have since Hamberger s (1784) work on the mechanical and geometrical considerations of respiration been generally held to be inspiratory in function This has been confirmed electromygraphically in man (Campbell 1958) and in this work actually also in the cat This deserves mention since it has been claimed recently by Eccles Sears and Shealy (1962) and by Sears (1964) that the internal intercostal nerve of the cat which innervates the internal intercostal muscle together with abdominal muscles only contains nerve fibres destined to expiratory muscles From the above it is clear that this is not the case and that the internal intercostal nerve contains fibres destined both for inspiratory *and* expiratory muscles This mis interpretation of Eccles et al may be the reason why they came to the somewhat strange conclusion that some *inspiratory* motoneurons are facilitated from *ex piratory* muscle spindles (see Sears 1966)

The effect of section of the dorsal roots

After careful rhizotomy the newborn did not show any change in the inter costal EMG activity in contrast to animals above 2 weeks of age where rhizotomy caused a considerable decrease in the activity This finding further supports the hypothesis that the muscle spindles lack gamma control and thus act as passive

stretch receptors only This result is in contrast to that obtained by Coombs and Pike (1930) who in kittens anesthetized with ether reported a decrease in thoracic respiration after rhizotomy Two possibilities for this inconsistency seem to exist either the ventral roots were also affected when the dorsal ones were cut or the method of recording was too crude for an adequate evaluation of thoracic movements Damage to ventral roots probably occurred sometimes during the present work as mentioned above Thus a negative result (i e no change in EMG) was held to be the most reliable As regards the method of recording intercostal activity used by Coombs and Pike they only reported that stethographs were placed around the thorax and abdomen" "to register costal and diaphragmatic respiration" The reported decrease in costal respiration on section of the thoracic dorsal roots is not documented by any published recordings Moreover their statement that the diminution of thoracic respiration in the newborn after section of the thoracic dorsal roots "may be of less significance than in adult animals" does in any case suggest that they also found that the thoracic dorsal roots were of relatively less importance in the newborn

Nerve fibre calibre measurements

It seems a general experience (see Skoglund and Romero 1965) that it is extremely difficult to obtain good quality preparations for nerve fibre measurements from newborn animals with the osmic acid method Although new and better methods are available which improve especially the quality of the smallest fibres (see Chapter V and VI) the classical method was used here with the purpose of obtaining results as comparable as possible with those from the lumbar region reported by Skoglund and Romero (1965) It should also be pointed out that due to the method used the proportion of fibres in the smallest group (1 21 μ—1 76 μ) is probably not so reliable Further some of the nerve preparations had to be discarded due to their bad quality (see page 66)

From the results of the fibre calibre analysis the following conclusions may be drawn Firstly from a comparison between the phrenic nerves and the thoracic ventral roots no evidence was found for any differences in maturity of the efferent fibres of these nerves i e there is no reason to believ that the preponderance of the diaphragmatic activity over that of the intercostal muscles in the newborn stage is due to a better developed efferent innervation of the former This comparison is valid since the analyses were made on nerves from the same animals and are thus not liable to the influence of individual variations It could be argued that the nerve fibres are not comparable since the phrenic nerves also contain afferent fibres This is true but it is probably not of significance in this connection since they amount to less than 10 % only and are mainly small (below 4—5 μ in the adult cat) (Hinsey Hare and Phillips 1939)

Secondly a peak of possible gamma fibres in the ventral roots was not found

until the age of 25 days However even at this stage this peak was absent in the intercostal nerves of the same animal Rexed (1944) also found this difference in man Since the same difference between the ventral roots and intercostal nerves was found in both cases (Th3 and Th6) it is probably not due to a methodological error Thus it is suggested that the peak of smaller fibres in the ventral roots consists mainly of preganglionic sympathetic fibres which make up the most important differences between the thoracic and lumbar ventral roots in the adult Thus a *real gamma" peak does not seem to appear until after 25 days supporting* the hypothesis that the intercostal muscle spindles lack gamma control during the earliest postnatal period

Thirdly as reported previously (Rexed 1944 Skoglund and Romero 1965) the dorsal root fibres were found to be slightly smaller than the ventral ones at birth since the relative amount of fibres above 3 42 microns was larger in the latter Further the fibre composition of the thoracic dorsal roots obtained here are rather *similar to those of the lumbar dorsal roots reported by Skoglund and Romero* (1965) Thus it might be concluded that the physiological properties of the thoracic afferents are comparable to the lumbar afferents and thus for instance unable to transmit impulses continually (Skoglund 1960 c) Certain objections can of course be raised against such a comparison since the thoracic and lumbar roots were taken from different animals and further different persons made the measurements However it is generally held that the thoracic region lags slightly behind the lumbar (and *cervical) region in development* at birth (Kingsbury 1932 Rexed 1944) Accordingly it seems highly unlikely that the thoracic afferents posses better functional properties than the lumbar afferents This then might further explain the poorly developed nervous control of the intercostal muscles found in the present work

Succinic dehydrogenase in the respiratory muscles

During postnatal development there was a marked increase in succinic dehydrogenase activity in the respiratory muscle fibres which were also found to differentiate with respect to this enzyme like the fibres of fast limb muscles (Nystrom 1966) The three kinds of muscle fibres found in the adult i e heavily stained lightly stained and intermediately stained could be identified already in muscles from 25 days old kittens This indicates that in this respect the respiratory muscles differentiate faster than hindlimb muscles which were reported by Nystrom (1966) not to be completely differentiated until 39 days postnatally With the method used here no evidence was found that the diphragm was in a more advanced state of development than the intercostal muscles in the newborn

With regard to the single twitch hind limb muscles are "slow" in kittens at birth but differentiate later some remaining "slow" others becoming fast" (Buller Eccles and Eccles 1960) The postnatal histochemical differentiation was by

Nystrom (1966) correlated to this physiological differentiation However it seems apparent that with regard to succinic dehydrogenase the most important change postnatally is that it *increases* in some of the muscle fibres Since the enzyme is mitochondrion bound this probably means that the number of mitochondria and thus the capability of aerobic metabolism increases and it might be suggested that "fatigue ensues faster in muscles with little enzyme activity This has been shown to be the case in muscle fibres of Xenopus Laevis (Lannergren and Smith 1966) Whereas tetanic tension fell in 30 sec to about 5 % of the maximal in pale muscle fibres with low enzyme activity it declined to about 70 % of the maximal only in dark and enzyme rich fibres Thus it appears reasonable to assume that the amount of oxidative enzyme is of importance for the ability to perform maintained actions This will be further taken up in the General Discussion

Summary

1) Changes in the activity of inspiratory intercostal muscles (recorded as the EMG) in response to various denervations and to tracheal occlusion were observed in cats and rabbits of different ages

2) Section of the vagi initially caused an increase in activity mainly due to a prolongation of the inspiratory phases Section of several pairs of thoracic dorsal roots subsequent to the vagotomy caused no or only a very slight decrease in the EMG activity of the newborn but a considerable decrease in animals above 3 weeks of age

3) Tracheal occlusion before inspiration caused a pronounced increase in the EMG activity during the subsequent inspiratory effort at all ages After section of the vagi no change or occasionally a slight decrease occurred in animals below some 10—15 days In older animals there was a marked increase After sectioning several thoracic dorsal roots bilaterally there was no change in EMG on tracheal occlusion at any age

4) The postnatal development of the fibre composition (calibre spectra) of the right 3rd and 6th thoracic dorsal and ventral roots and intercostal nerves and of the right phrenic nerve was studied in osmium stained 3—5 μ sectioned material

5) At birth the ventral root fibres were found to be slightly larger than the dorsal ones At 25 days the fibre calibre spectra of these roots showed two peaks one at about 2 μ and the other at about 5—6 μ In the intercostal nerves of the same segment there was only a single peak at about 5—6 μ Accordingly it was concluded that the peak of small fibres in the ventral roots consists of sympathetic preganglionic fibres and that the gamma fibres do not contribute to this peak to any greater extent until after this age

6) The growth of the phrenic nerves was very similar to that of the thoracic ventral roots

7) It is concluded that the nervous control of the intercostal muscles is poorly developed at birth in cats and rabbits which might partly be explained by a relative immaturity of the thoracic dorsal root afferents and partly by a lack of "gamma" control of the intercostal muscle spindles

8) Postnatally there is a considerable increase in succinic dehydrogenase activity in the intercostal muscles and the diaphragm Further the fibres of these muscles differentiate into three different types with regard to the activity of this enzyme as do "fast limb muscles This differentiation is qualitatively completed 25 days after birth *The significance of this enzyme is discussed briefly*

Ventilatory reactions to artificial changes of blood pCO_2 and pH and blood carbonic anhydrase activity during postnatal development in the cat

Introduction

There seems to be general agreement that a moderat increase in the partial pressure of CO_2 in the respired air augments ventilation in newborn infants and animals (see Purves 1966 c) It is therefore of note that the increase in pCO_2 and decrease in pH in the arterial blood which occurred in newborn kittens after section of the vagus nerves did not stimulate respiration since CO_2 progressively accumulated during the test period (see page 29 Fig 5) This was not the case in the adult animal where the arterial pCO_2 and pH values remained constant after vagotomy It may thus be questioned if the central chemoreceptors" are not functionally developed in the newborn and if the ventilatory augmentation caused by an increase in CO_2 of the respired air is due to a stimulation of the peripheral chemoreceptors only Purves (1956 c) reported that newborn lambs increased their ventilation in response to CO_2 even after sinus nerve section and suggested that this response was due to a stimulation of the "central chemoreceptors" In this case however a possible influence from aortic and/or pulmonary chemoreceptors cannot be excluded since the vagus nerves were intact

This chapter deals with the ventilatory reactions to artificially induced changes in blood acid base balance during postnatal development in the cat The following questions were posed

1) Is the ventilation of the newborn animal augmented by an increase of CO_2 in the respired air? If so can this be partly attributed to a stimulation of the "central chemoreceptors"?

2) Does an infusion of an acidifying solution stimulate respiration and an infusion of a base inhibit it in the newborn as in the adult?

Berfenstam (1952) found that the blood carbonic anhydrase activity increased during postnatal development in infants It was therefore decided to test whether

the activity of this enzyme also increased in the cat during postnatal development and if so when the adult value was reached.

Material and methods

59 experiments were carried out on 39 kittens aged 1—8 days, 7 kittens 10—21 days, 4 kittens 28—40 days and 9 cats from 6 weeks to fully grown. Most animals were lightly anesthetized with Nembutal while the remainder were decerebrated; details are given below.

A tracheotomy was performed as a routine. Respiration, arterial pH and pCO and blood pressure were recorded (p. 18). Gas mixtures containing 3 % and 6.5 % CO_2 in air were administred for periods of 5—15 minutes as described earlier (p. 21). As acidifying solutions either 0.5 or 0.05 N HCl or NH_4Cl were used. As bases either 1.0 M $NaHCO_3$ (freshly prepared and used immediately to minimise formation of Na_2CO_3) or 1.0 M Na_2CO_3 solutions were used. As a rule 1 ml/kg b.w. was injected intravenously at a moderate rate, i.e. during a 10—20 seconds period via the right three-way stop-cock shown in Fig. 2, page 20. As a control an equal amount of Ringer's solution was injected; this never affected respiration.

The effect on the vagal afferent activity in response to injections of acids and bases were studied in 5 (Nembutal) anesthetized kittens (3 aged 1—4 days, 2 aged 30 and 40 days). A wide thoracotomy was performed and the animal was ventilated with a respirator (Model RU 4M, ENSCO). A constant volume of air was inflated into the lungs so the distension of the pulmonary stretch receptors was the same at each inflation. Recordings were made from thin filaments of the distal end of one cut cervical vagus nerve as described in detail on page 35.

The carbonic anhydrase activity was estimated according to the colorimetric method of Philpot and Philpot (1936) in whole blood withdrawn from 33 Nembutal anesthetized cats and kittens (1 day—adult). Thus the time required for the cytolysed and denatured whole blood diluted 1/20 to lower the pH of a CO_2 saturated carbonate solution from about 10.5 to 7.0 was measured. Due to the small volume of blood that could be withdrawn from the youngest animals only one measurement was made in each case. Bromthymol blue was used as the indicator and distilled water as a blank.

Results

I. *The response to administration of 3 % and 6.5 % CO_2 in spontaneously respiring cats*

a) Peripheral chemoreceptors intact

The general finding was that animals of all ages from birth upwards responded with increased ventilation when breathing air containing 3 % or 6.5 % CO_2

which is in accord with earlier findings (see Chapter I) As noted previously (p 17) however kittens below 2—3 weeks of age were particularly sensitive to the anesthetic and if too deeply anesthetized did not increase ventilation in response to CO_2 in the respired air (Fig 1 p 17) Very lightly anesthetized (20—25 mg Nembutal/kg b w) kittens below 2—3 weeks however were also often found to accumulate CO_2 progressively and the initially increased ventilation diminshed This was most often the case when 6 5 % CO_2 was administred Thus whereas most animals of this age group tolerated 3 % CO_2 their ventilation began to decline after respiring 6 5 % CO_2 for a few (3—5) minutes

Fig 34 shows the changes in arterial pCO_2 and pH and in ventilation when a 4 months old (A) and a 6 days old (B) cat are exposed to 3 % CO_2 in the respired air During the first minutes there was a progressive increase in arterial pCO_2 and decrease in pH and a simultaneous increase in ventilation After some 2—4 minutes a new steady state was reached The corresponding changes taking

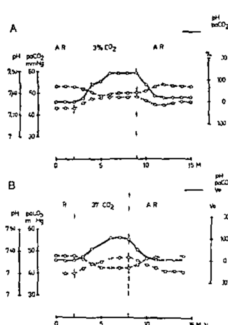


Fig 34 Diagrams showing the effect of administration of 3 % CO_2 in the respired air on ventilation and arterial pH and pCO_2 in a 4 months old Nembutal anesthetized (40 mg/kg b w) cat (A) and a 6 days old decerebrate kitten (B)

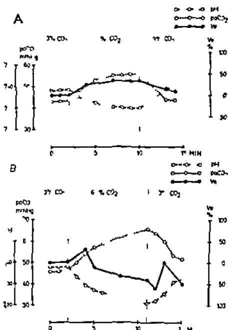


Fig 35 Diagrams showing the effect of administration of 6 5 % CO_2 in the respired air on ventilation and arterial pH and PCO_2 in a 3 months old Nembutal anesthetized (40 mg/kg) cat (A) and a 6 days old Nembutal anesthetized (30 mg/kg) kitten (B) The animals were breathing 3 % CO_2 in air before the administration of 6 5 % CO_2

place when a 3 months old and a 6 days old cat were exposed to 6 5 % CO are shown in Fig 35 As can be seen ventilation increased in the older animal and a new steady state was reached within a few minutes In contrast the ventilatory respon e of the younger animal was biphasic and after an initial augmentation began to decline The arterial pCO_2 increased progressively while the pH decreased The pH and pCO_2 values at which ventilation began to decline are seen to be about the same as those at which the older animal continued hyperventilating in spite of the fact that the latter was given relatively more Nembutal A record of respiration from a 4 days old kitten when exposed to 6 5 % CO_2 in the respired air is shown in Fig 36 It can be seen that the ventilatory augmentation is characterized both by an increase in frequency and amplitude whereas the depression is characterized by a decrease in both Sometimes a gasp like breathing pattern ensued Ventilation was also eventually depressed by 6 5 % CO_2 in decerebrate kittens below 2—3 weeks of age although the duration of the hyperventilation was regularly longer In a few cases the arterial pCO_2 and ventilation decreased below the respective original values when CO_2 was again replaced by air This indicates that the endogenous CO production and thus the aerobic metabolism had been depressed during CO breathing

b) Peripheral chemoreceptors denervated

Shortly after section of the vagus and sinus nerves the administration of 3 % CO_2 in the respired air caused a marked respiratory stimulation as illustrated in

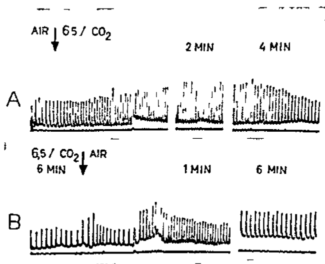


Fig 36 Respiratory record (intraoesophageal pressure swings inspiration deflection upwards) from a 4 days old Nembutal anesthetized (30 mg/kg) kitten Administring 6 5 % CO_2 in the respired air (A) caused a transient increase and a secondary decrease of respiration Replacing 6 5 % CO_2 by air (B) initially augmented respiration again

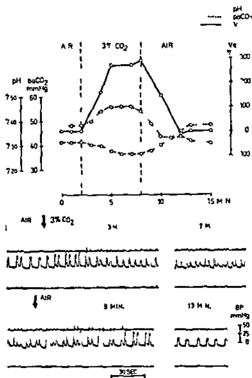


Fig 37 The effect of administration of 3 % CO_2 in the respired air shortly after denervation of the peripheral chemoreceptors Kitten _ days old anesthetized with 20 mg/kg Nembutal carotid arteries ligated The diagram shows the changes in ventilation and arterial pH and pCO The records show respiration (intraoesophageal pressure swings inspiration deflection upwards) and blood pres ure

Fig 37 As can be seen there was both an increase in respiratory f equency and amplitude Since all known peripheral chemoreceptors were denervated this response can probably be attributed to a stimulation of the "central chemoreceptors" The increase in amplitude is very marked which may be explained by the lack of vagal inhibition i e the "Hering Breuer inflation" reflex (cf Chapter V) This might be the reason why the relative increase in ventilation can be as much as 300 % compared to about 100 % in the animals where the vagus nerves are intact (see above) If an animal was exposed to 3 % CO_ between half an hour and an hour after the denervation there was as a rule no increase in respiration and sometimes it was only further depressed The circumstances under which a depression in response to CO_ becomes preponderant after vagotomy was not however analyzed in further detail

II *The response to acids and bases*

a) Acids

Injection of 1 0 ml/kg b w of a 0 5N HCl or NH_4Cl solution into cats and kittens above 1 week of age always gave rise to a pronounced initial respiratory stimulation as shown in Fig 38 After some 5 minutes the pH became stabilized at some 0 04—0 05 units below and ventilation at some 75 % above the original values In animals below 2 weeks of age the same relative amount of acid also most

often stimulated respiration but affected it initially more seriously as seen from Fig 39 Thus during the first 2 minutes a gasp like type of breathing appeared whereby CO_2 accumulated and the pH fell more than in the older animals Some 3 minutes after the injection however ventilation increased again and later stabilized at some 90 % above the original value At that stage the arterial pH was some 0 05 units below and pCO at the original value which thus indicates that a fall in blood pH does stimulate respiration also in the newborn

The difference in the initial response indicates a more pronounced susceptibility to acidification of the blood in the newborn Sometimes respiration never improved after the initial change in response to the injection of the acid but remained depressed If the concentration of the HCl or NH_4Cl solution was lowered ten times i e to 0 05N an injection of 1 0 ml/kg b w into a kitten below 2 weeks of age often gave rise to such a ventilatory augmentation that the arterial pH increased after a small initial fall and the pCO_2 decreased after a small increase Such an experiment is illustrated in Fig 40

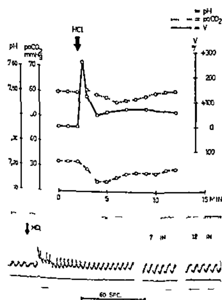


Fig 38 The effect of an i v injection of 1 0 ml/kg of a 0 5 N HCl solution into an adult cat anesthetized with 40 mg/kg Nembutal The diagram shows the changes in ventilation and arterial pH and PCO_2 The records show respiration (intraoeso phageal pressure swings inspiration de flection upwards)

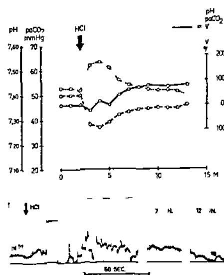


Fig 39 The effect of an i v injection of 1 0 ml/kg of a 0 5 N HCl solution into a 2 days old kitten anesthetized with 30 mg/kg Nembutal Diagram and records as in Fig 38

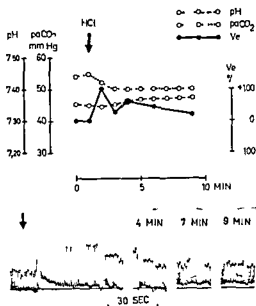


Fig 40 The effect of an i v injection of 1 0 ml/kg of a 0 05 N HCl solution into a 1 day old kitten anesthetized with 35 mg/kg Nembutal Diagram and records as in Fig 38

b) Bases

Animals above 4 weeks of age regularly responded to an injection of 1 0 ml/kg b w of a 1 0 M Na_2CO_3 or $NaHCO_3$ solution in the same way as adults i e by a decrease in ventilation When $NaHCO_3$ was injected a characteristic initial ventilatory increase was seen before ventilation became stabilized at a level below the original value (Fig 41) This initial stimulation has been attributed to the production of CO_2 which is the immediate consequence of the increase in HCO_3 ions (Singer Deering and Clark 1956) Respiration was depressed by an injection of a base in animals above 4 weeks of age also when they were highly acidotic (Fig 41)

The first injection of a base into an animal below 2 weeks of age was most often a pronounced ventilatory augmentation (Fig 42) This "paradoxical" responce consequently lowered the arterial pCO_2 A second injection of equal size on the other hand regularly gave the adult type" of response (Fig 42)

A respiratory stimulation in response to an injection of a base was most often also seen in decerebrate animals below 2 weeks of age Only in a one such animal did the first injection give a sustained respiratory inhibition but unfortunately in this case the blood acid base parameters were not recorded simultaneously The first injection of a base given shortly after vagotomy also stimulated ventilation Finally a base caused a paradoxical augmentation of ventilation when injected into animals hyperventilating during breathing of 3 % CO_2 (Fig 43)

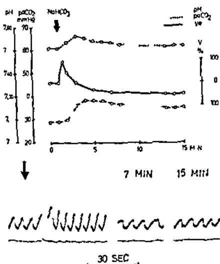


Fig 41 The effect of an i v injection of 1 0 ml/kg of a 1 0 M $NaHCO_3$ solution into an adult cat anesthetized with 40 mg/kg Nembutal Diagram and records as in Fig 38

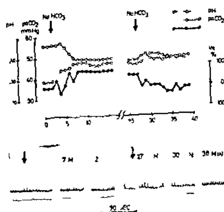


Fig 4 The effe t of successive i in jections of 1 0 ml/kg of a 1 0 M $NaHCO_3$ solution into a kitten 2 days old anesthe tiz d with 30 mg/kg Nembutal Diagram and records as in Fig 38

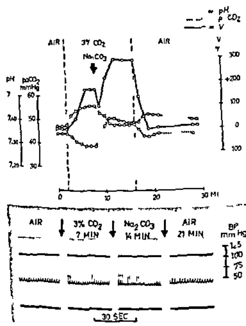


Fig 43 The effect of an i v injection of 1 0 ml/kg of a 1 0 M Na_2CO_3 so lution into a kitten 10 days old (anes thetized with 30 mg/kg Nembutal) breathing 3 % CO_2 in air Diagram and records as in Fig 38

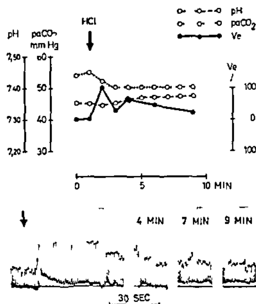


Fig 40 The effect of an i v injection of 1 0 ml/kg of a 0 05 N HCl solution into a 1 day old kitten anesthetized with 35 mg/kg Nembutal Diagram and records as in Fig 38

b) Bases

Animals above 4 weeks of age regularly responded to an injection of 1 0 ml/kg b w of a 1 0 M Na_2CO_3 or $NaHCO_3$ solution in the same way as adults i e by a decrease in ventilation When $NaHCO_3$ was injected a characteristic initial ventilatory increase was seen before ventilation became stabilized at a level below the original value (Fig 41) This initial stimulation has been attributed to the production of CO_2 which is the immediate consequence of the increase in HCO_3 ions (Singer Deering and Clark 1956) Respiration was depressed by an injection of a base in animals above 4 weeks of age also when they were highly acidotic (Fig 41)

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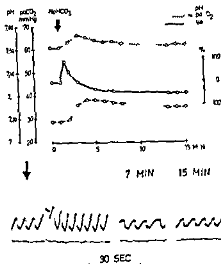


Fig 41 The effect of an i v injection of 1 0 ml/kg of a 1 0 M $NaHCO_3$ solution into an adult cat anesthetized with 40 mg/kg Nembutal Diagram and records as in Fig 38

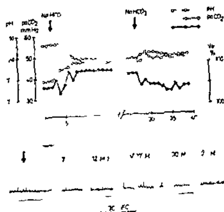


Fig 4 The ffe t of successive i v in jections of 1 0 ml/kg of a 1 0 M $NaHCO_3$ solution into a kitten 2 days o d anesthe tized with 30 mg/kg Nembutal Diagram and records as in Fig 38

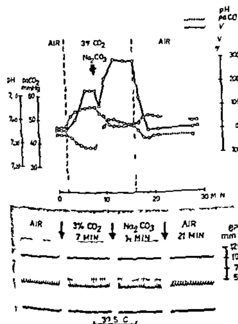


Fig 43 The effect of an i v injection of 1 0 ml/kg of a 1 0 M Na_2CO_3 solution into a kitten 10 days old (anesthetized with 30 mg/kg Nembutal) breathing 3 % CO_2 in air Diagram and records as in Fig 38

A respiratory stimulation very similar to that caused by a ba e could be induced by a painful stimulus (pinching the tail) (Fig 44 B) This was most marked in young animals below 2 weeks of age though a slight response could be induced in adults (Fig 44 C)

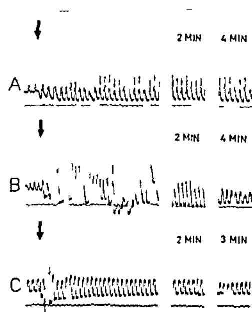


Fig 44 Respiratory records (intraoesophageal pressure swings inspiration deflection upwards) from an 8 days old Nembutal anesthetized (30 mg/kg) kitten (A B) and an adult decerebrate cat (C) In (A) 1 0 ml/kg of a 1 0 M Na_2CO_3 solution is injected (arrow) In B and C a painful stimulus is set up (pinching the tail arrows)

III *The effect on the vagal afferent activity in response to acids and bases*

The augmentation of ventilation in animals below 2 weeks of age may be due to a possible stimulation by the acids and bases on the peripheral input The afferent activity in small filaments of the peripheral end of one vagus nerve was therefore recorded in 5 kittens during intravenous infusions of acids and bases This nerve was chosen since its normal discharge pattern is known (Chapter V) When 1 0 ml/kg b w of a 0 5 N HCl solution was injected the e was no visible change in the activity but with 1 0 ml/kg b w of a 1 0 M Na_2CO_3 on the other hand there was a prorounced increase in the discharge in animals of all ages (Fig 45) From the record (Fig 45) it appears that both the frequency or earlier active units increased and that new units were recruited Not until 5—7 minutes after the injection did the discharge return to the pre injection level While one or two subsequent injections gave the same response in the animals above 30 days of age in 1 -4 days old animals a second injection gave rise only to an initial increase in discharge activity after which a considerable decrease occurred (Fig 46) In one case the discharge from a single pulmonary st etch unit changed its pattern completely it ceased firing synchronously with respiration and sho ed short successive bursts of activity before it finally failed completely (Fig 46)

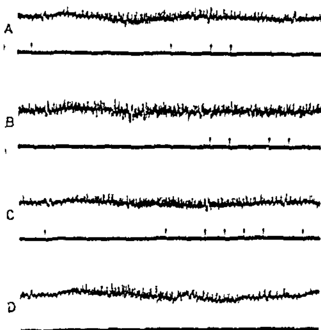


Fig 45 Recordings from a thin filament of the left vagus nerve in a 40 days old kitten (A) before (B) 2 min, (C) 4 min (D) 7 min after an i v injection of 1 0 ml/kg of a 1 0 M Na CO_3 solution Animal thoracotomized, artificially ventilated

It may thus be concluded that the injection of a base appears to increase the discharge from the pulmonary stretch receptors in cats of all ages Probably also other kinds of receptors increased their discharge also as seen from the records However although this was the case in response to repeated injections in the older animals the receptors or the receptorafferents of the newly born appeared to be more susceptible and to deteriorate functionally already after a second injection of an equal dose of Na CO_3

IV *Blood carbonic anhydrase activity*

In Fig 47 is plotted the time required for the whole blood preparation from cats and kittens of various ages to lower the pH of a carbonate solution from 10 5 to 7 0 as indicated by a sharp change in colour of the bromthymol blue from blue to yellow green The time required was about 40 sec for blood from new born animals This time then decreased with increasing age and the adult value i e about 15 sec was reached in 3—4 weeks old animals The time required for the water blank was 58—72 sec (5 measurements) Thus there is a pronounced increase in the blood carbonic anhydrase activity during postnatal development of the cat the adult value being reached at 3—4 weeks of age

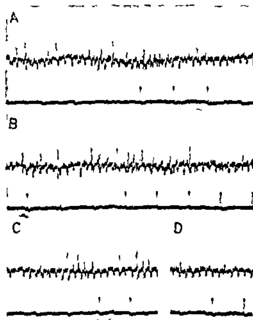


Fig 46 Recordings from a thin filament of the left vagus nerve in a 1 day old kitten (A) before (B) 1/2 min (C) 3 min (D) 7 min after a second i v injection of 1 0 ml/kg of a 1 0 M Na_2CO_3 solution Animal thoracotomized artificially ventilated Time 100 msec

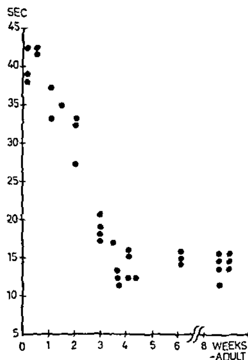


Fig 47 Time required for cytolysed denatured whole blood preparations from cats of different ages to lower the pH of a carbonate solution from about 10 5 to 7 0 (see text)

Like newborn infants and lambs (see Chapter I) newborn kittens were found to increase their ventilation in response to an increase in CO_2 of the respired air Since this also occurred after cutting the vagus and sinus nerves which denervates all known peripheral chemoreceptors the central chemoreceptors appear to be functioning in the newborn No attempt was made to compare quantitatively the ventilatory response to CO_2 in the newborn with that of the adult or to compare the relative importance of the central and peripheral chemoreceptors in this respect

In accordance with earlier reports on newborn infants (Stahlman 1961 Avery Chernick Dutton and Permutt 1963) newborn kittens were however also found to be easily depressed by CO_2 and to accumulate it progressively Thus when 6 5 % CO_2 was administrated the ventilatory response was regularly biphasic, i e the initial increase was followed by a decrease and a consequent accumulation of CO Since CO_2 appears to cause bronchoconstriction (see Widdicombe 1963) the pulmonary compliance will decrease (Schwieler 1966) and the ventilation will be overestimated (cf Chapters IV and VI) and thus the real ventilatory decrease will be larger than that calculated This ventilatory depression may be partly attributed to a greater susceptibility of the newborn to the anesthesia The depression however occurred at about the same pH and pCO_2 values at which the 3 months old animal maintained hyperventilation in spite of the fact that it had received more Nembutal (p 89) Also adult animals are depressed by high levels of CO_2 (see Gray 1950) but this CO_2 reversal thus appears to occur at a relatively higher blood pCO than in the newborn In Chapter IV (p 28) it was found that section of the vagus and sinus nerves caused a decrease in ventilation and an increase in pCO in animals below 2—3 weeks From the above it may be concluded that the respiration of the newborn thereby became further depressed This might partly explain why administration of CO_2 half an hour to an hour after the denervation did not stimulate respiration (p 90)

The CO_2 induced respiratory depression was characterized both by a decrease in amplitude and frequency and was very similar to the depression caused by hypoxia (see Fig 24 p 57 and Fig 36 p 89) As in hypoxia sometimes also gasping occurred in the youngest (1—2 weeks) animals on exposure to CO and in a few cases evidence was found that the endogenous production of CO and thus the aerobic metabolism decreased In adult animals very high concentrations of CO (10—15 %) in the respired air is known to cause a reduction in oxygen consumption (see Holmdahl 1956) and it seems possible that the amount of CO required to cause a decrease in the aerobic metabolism is smaller in the newborn This may be explained by the fact that the number of mitochondria in for example the nervous system is relatively less in the newborn than in

the adult (Samson Balfour and Jacobs 1960 Skoglund 1967 b) The possible relationship between the ventilatory depression and decrease in oxidative metabolism during hypoxia and hypercapnia will be discussed later (p 105)

The effects of injections of acids and bases into the blood might suggest that the nervous system of the newborn is more susceptible than that of the adult to artificially induced blood acid base changes Since the amount of acid or base that was injected was constant relative to body weight at all ages and since the relative volumes of blood (Mott 1965) and extracellular fluid (Friis Hansen 1957) decrease postnatally the newborn animals received in fact relatively less acid or base than the adult Moreover since the results obtained from animals above 4 weeks of age were in general the same as those from adults (see also Hesser 1949) it appears unlikely that the results from the youngest animals can be ascribed to inconsistencies of the methods used

While an injection of an acid solution most often caused a ventilatory stimulation both in the newborn and adult animals respiration was initially more seriously affected and gasping often occurred in the former If in analogy with newborn infants (Bland 1956) the capacity of the blood buffer system (the bicarbonate and phosphate systems serum protein and hemoglobin) is also lower in the new born cat this might result in a lower pH in the central nervous system after the injection But since a tenfold reduction of the concentration of the acid solution often stimulated respiration to such an extent that the arterial pH increased it appears that either the respiratory neurons are more labile and susceptible to acid base changes or the H or HCO_3 ions penetrate the "blood brain barrier" more easily than in the adult

According to Leusen (see Leusen 1965) a metabolic" alkalosis or acidosis does not give rise to any appreciable change in the pH of the cerebro spinal fluid (CSF) A respiratory" blood acid base change on the other hand is rapidly followed by a corresponding change in the CSF Accordingly it has been argued that CO_2 as such easily diffuses through the blood brain or blood CSF" barrier of the adult while the diffusion velocity of HCO_3 ions is much lower so that variations of the blood [HCO_3] are not immediately followed by corresponding changes in CSF

There is evidence that the "blood brain barrier" is less effective in newborn animals Although certain criticism has been directed towards this statement (see Dobbing 1961 1963) Dempsey and Luse (1958) and Pappas and Purpura (1964) from electron microscopic investigations report that the neurons of the CNS lie in direct contact with the vessels neonatally whereas at later developmental stages they are well separated from these by intervening glial and dentritic processes However it must be stressed that even pain provoked for example by pinching the tail also gave a very strong ventilatory stimulation of similar

pattern and it could be argued that the result of the HCl injection is due to some diffuse noxious stimulation such as via the vascular bed However the effect of an acid on the peripheral input does not seem to be as pronounced as that of a base (see below)

Generally the first injection of a base into a newborn animal quite unexpectedly produced a marked augmentation of ventilation i e a "paradoxical response with a consequent CO_2 wash out This occurred in most decerebrate animals in vagotomized animals and in animals where respiration had been stimulated by administering 3 % CO_2 in the respired air On the other hand a second injection most often gave an "adult" response viz a ventilatory diminution and a consequent CO_2 accumulation

It was pointed out in Chapter I (p 14) that the general effect on the CNS of an alkalinization of the blood was activation and increased excitability Only the neurons of the reticular activating system or alternatively some medullary chemo sensitive structure were activated by increasing acidity If it is assumed that the injection of a base activates the respiratory effector neurons of the CNS which in the beginning of the experiment might have been relatively depressed due to a greater susceptibility to acidosis the outcome of the effector system in response to the same or even smaller "receptor" stimulation will be greater That neural function in general really is activated by an alkalinization of the blood has been repeatedly confirmed (see Wyke 1963) and was supported here by the results from the recordings of the vagus afferents which increased their discharge in response to an injection of Na_2CO_3 Since not only the pulmonary tension receptors but also other vagal afferents increased their discharge it might be assumed that the injection of the base produces a marked increase in the diffuse afferent input on the CNS which might partly be the cause of the ventilatory increase analogous to the effect of pinching the tail (Fig 44) Although the initial effect of injecting Na_2CO_3 was sometimes a respiratory stimulation (Fig 44 A) there was most often a slight ventilatory diminution (Fig 43) Part of this inhibition might very well be explained by the increase in discharge from the pulmonary tension receptors which normally inhibit inspiration But since it occurred also after vagotomy it might be attributed to some central effect as for example an inhibition of the reticular activating neurons or alternatively the central chemoreceptors When stimulation was the immediate effect the exciting diffuse input might have been dominant together with the increased ability of the respiratory system (see above) This will be further discussed later (p 106)

The receptors or the afferent vagus nerve fibres in newborn animals seem to be more susceptible to the Na_2CO_3 injection since they ceased or decreased dis charging or completely changed their firing pattern after a second injection This was never seen in the 30 and 40 days old animals It might indicate that the reason for the adult ventilatory response to the second Na_2CO_3 injection is

caused by impaired neural function in the newborn and not as in the adult by an inhibition of the central chemoreceptor mechanism Again this appears to be due either to a greater susceptibility of the neurons themselves or to the fact that they are less well protected from changes in the blood by a barrier It has been proposed that in the adult a functional barrier similar to that between the vascular bed and the central neurons is present between the vessels and the extra cellular space around the nerve fibres (see Dobbing 1961)

The method used here to measure the blood carbonic anhydrase activity has been much criticized (see Davis 1961) Thus the results obtained will not be used for any quantitative calculations which could further throw some light on the results obtained with regard to the reactions of the artificially induced blood acid base changes in the newborn It is noteworthy, however that the adult carbonic an hydrase value is reached about 4 weeks after birth when also the reactions of the animals were adult This requires further study

Summary

1) The ventilatory response to administration of CO_2 in the respired air and to i v infusions of acids and bases have been studied in cats and kittens of various ages from birth upwards Intrathoracic pressure changes arterial pH and pCO_2 and blood pressure were recorded

2) As in the adult respiration in the newly born was found to be stimulated by a moderate increase in CO_2 of the respired air However whereas 3 % CO_2 most often gave rise to a maintained hyperventilation in animals of all ages the response to 6 5 % CO_2 was regularly biphasic in animals below 2 weeks of age after an initial augmentation ventilation diminished with a consequent progressive CO_2 accumulation

3) A moderate increase in CO_2 of the respired air stimulated respiration in the newly born also shortly after section of the vagus and sinus nerves which indicates that the central chemoreceptor mechanism is functionally active at birth

4) An i v injection of 1 0 ml/kg of a 0 5 N HCl or NH_4Cl solution into animals above 4 weeks of age always caused a marked respiratory stimulation In animals below 2 weeks of age the same weight dose most often caused an initial respiratory depression and only a secondary stimulation Sometimes how ever the depression was prepondering If the concentration of the acid solution was reduced ten times injection of an equal amount caused an overcompensation i e ventilation increased to such an extent that the arterial pH rose and pCO_2 fell

5) Injecting 1 0 ml/kg 1 0 M $NaHCO_3$ or Na_2CO_3 caused a ventilatory dim inution in animals above 4 weeks old (after a short initial augmentation when $NaHCO_3$ was injected) A first injection of an equal relative amount of a base into animals below 2 weeks of age most often caused a ventilatory *augmentation* whereas a second injection depressed ventilation

6) One injection of 1 0 ml/kg 1 0 M Na_2CO_3 caused a marked increase in the vagal afferent discharge Whereas this was the case also in response to one or two successive injections in animals 30—40 days old a second injection only caused an initial increase and a secondary decrease or complete cessation of the discharge in animals 2—4 days old

7) Blood carbonic anhydrase activity was found to increase considerably during postnatal development the adult value being reached at the age of 3—4 weeks

General discussion

The results of the present work clearly show that important differences exist between the newborn and adult animal with regard to the control of respiration The concept generally held that once respiration has started in the newborn it is controlled in the same way as in the adult (see James and Adamsons 1964 a b Cross 1965) must therefore be revised Although the pulmonary tension receptor afferents were found to be relatively mature and the "Hering—Breuer inflation" reflex was present in the newborn (Chapter V) the influence of the thoracic dorsal root afferents on the regulation of respiration appeared negligible (Chapter VII) This can probably be attributed to a lack of "gamma" control of the intercostal muscle spindles and to a relative immaturity of their afferents The peripheral chemoreceptors (Chapter VI) were functionally active in the newborn and influenced respiration essentially as in the adult but the oxygen partial pressure range within which they were effective appeared to be smaller Thus in contrast to adult animals the ventilatory response to 10 % O_2 was biphasic an *initial augmentation was followed by a depression in spite of a continuous* and even increasing receptor discharge The central chemoreceptors" were also found to be functionally active since an increase in pCO_2 of the inspired air augmented ventilation even after denervation of all known peripheral chemoreceptors However the newborn was also more easily depressed by hypercapnia and the ventilatory response to 6 5 % CO_2 was regularly biphasic with an initial increase and a subsequent depression The reaction of the newborn to injection of acids and bases finally revealed a greater sensitivity to such artificially induced acid base changes than in the adult animal and a greater lability of the respiratory center Whereas in adults acid had a stimulatory effect an injection of 1 ml 0 5 N HCl/kg body weight in the newborn tended to depress ventilation at least initially while 0 05 N HCl often produced overcompensation with hyperventilation Moreover the injection of a base most often caused a "paradoxical response viz an increase in ventilation and a consequent CO wash out

A general finding was that by about 4 weeks of age the reactions of the respiratory system had become adult as had the effects of vagotomy The different control systems develop gradually at different relative rates Thus a load compensating mechanism of the intercostal muscles could be seen in kittens at about 10 days old and in rabbits at about 15 days old although the strength of this reflex probably increased further subsequently The postnatal diminution of the dominance of the paradoxical reflex" of Head is difficult to establish and requires further studies for a firm statement to be made The reaction to artificial blood acid base shifts were generally of the mature pattern in cats between 3 and 4 weeks whereas a biphasic response to hypoxia was sometimes

also seen in 4 weeks old animals There is probably some interaction between the different control systems of different relative maturity and it was found to be very difficult to define the precise time at which a certain control system became fully developed Individual variations in maturation may also complicate this analysis

The observation by Coombs and Pike (1930) that vagotomy affects respiration more in the newborn was confirmed in the present work (Chapter IV) The following factors probably contribute to this effect 1) an absence of control from the thoracic dorsal root afferents in the newborn 2) a greater susceptibility to the hypoxia and hypercapnia which are consequences of the decreased ventilation after vagotomy 3) the withdrawal of a facilitation from the arterial chemo receptors and 4) of other facilitatory pulmonary receptors such as those responsible for the paradoxical reflex of Head The relative importance of the different factors are impossible to estimate and furthermore 5) the withdrawal of inhibition from the relatively mature pulmonary tension receptors might have a different effect on the newborn compared with that in the adult

A prolongation of the inspiratory phases following vagotomy was observed at all ages The consequent decrease in respiratory frequency however appeared to be more pronounced in the newborn which partly explains the reduction of ventilation Moreover especially in decerebrate animals vagotomy sometimes caused a gasp like or periodic respiration the latter similar to Biot s or Cheyne Stoke s breathing Since such breathing patterns generally reflect in coordination of the respiratory control system (see Hoff and Beckenbridge 1954) the vagi thus seem to be more important for coordination of respiratory activity in the newborn But it also seems possible that the reason for the periodic or gasp like breathing might be that the respiratory neurons become exhausted when lacking inhibition Thus the inspiratory neurons appear to fire maximally for a short while thereby becoming exhausted to recharge during the pause and then to discharge maximally for a short period again This may in fact be a common feature of immature neurons since essentially the same phenomenon was observed by Skoglund (1960 c) and Ekholm (1967) in muscle and skin afferents respectively

The possibility that the vagal inhibition is more important in the newborn needs some further comment Widdicombe (1961) claimed that the strength of the Hering Breuer inflation reflex decreases from rabbits and rats through cats and dogs to man in the mammalian series Moreover in amphibia the most im portant afferent control of the respiratory movements seem to be mediated via the vagi (for references see Taglietti and Casella 1966) whereas in the adult cat the thoracic afferents seem to be of about equal importance as those of the vagi in this respect (Euler and Fritts 1963) Thus phylogenetically the development of

thoracic respiration appears to reduce the importance of the vagal afferents for respiratory control From the results of the present work this appears to be true also for ontogenetic development since the importance of the thoracic dorsal roots appears to be negligible at birth and to develop postnatally in the kitten and rabbit Such a development might also in part explain why Cross et al (Cross Klaus Tooley and Weisser 1960) found that the "Hering Breuer inflation reflex was stronger in the newborn infant than in adult man The theory of "Selbststeuerung of respiration (see p 10) by the vagal afferents (Hering 1868 Breuer 1868) thus seems to be more applicable to the newborn

As pointed out above the decrease in ventilation after vagotomy might be attributed partly to a disappearance of the facilitation exerted by different vagal afferents (see Comroe 1964 Widdicombe 1964) Evidence was found that the peripheral chemoreceptor afferents are relatively mature in the newborn and that they continuously facilitate respiration during air breathing since administration of pure oxygen stopped their discharge with a consequent ventilatory diminution However also the affe ents which on stimulation facilitate inspiration and give rise to the so called "Head s paradoxical reflex are probably of importance in this respect Whereas this reflex is very easy to produce in fullterm fetal or new born lambs as well as in the newborn infant (Cross Klaus Tooley and Weisser 1960 Hughes Parker and Williams 1967) a partial vagal block is necessary in the adult (Widdicomb 1967) This facilitatory influence thus appears dominant in the newborn and becomes concealed in the Sherringtonian sense (see Creed et al 1932) during postnatal development An interesting parallel might here be drawn to the finding by Ekholm (1967) that the dominating excitatory influence from the skin in the early developmental stages becomes concealed with increasing age

While both the inhibitory and facilitatory (and of these especially those re sponsible for the paradoxical reflex of Head) afferents conveyed via the vagi thus appear to be dominant for respiratory control in the newborn those of the thoracic dorsal roots appear to be of minor importance (Chapter VII) Section of the dorsal roots did not change the intercostal muscle activity appreciably in the newborn and a reflex "load compensating mechanism (see Corda Eklund and Euler 1965) could not be demonstrated until some 10—14 days after birth Based on these findings and on fibre calibre measurements of the thoracic dorsal and ventral roots and intercostal nerves it was concluded that the intercostal muscle spindles lack gamma" control and that their afferents are relatively im mature Thus when the vagi are sectioned the most important control of the interco tal muscles and the diaphragm (Decima Euler and Thoden 1967) is withdrawn since there is no control from the thoracic afferents This finding contributes to the understanding of the important role of the vagi in the newborn

The lack of "gamma" control of the intercostal muscle spindles may have further implications on the regulation of respiration since Euler and Soderberg (1952) and Burns (1963) have shown that in adult animals the diffuse input from receptors in all parts of the body is necessary for the respiratory neurons to maintain activity In the newborn the discharge from muscle spindles not being "gamma" driven must be extremely meagre Skoglund (1960 c) also showed that in the newborn at least the distal hindlimb muscle spindles lack gamma control In combination with Ekholms (1967) finding that also the discharge from other somatic receptors (skin articular) is meagre neonatally this infers that the newborn may have a deficient diffuse input This might explain why interruption of the vagi with their relatively mature facilitatory afferents has so deleterious an effect

It was found (Chapter VI and VIII) that respiration in the newborn was more sensitive to hypoxia and hypercapnia The depression due to hypoxia was essentially a central effect and was not mediated through the peripheral chemoreceptors which fired continually The reason why this discharge becomes ineffective might be firstly that the central afferent terminals become blocked due to hyperpolarization as do immature spinal muscle afferents when firing at high frequencies (Skoglund 1960 d) or secondly the hypoxia might in some way change the threshold of the respiratory neurons for the receptor discharge since the continual and even increasing chemoreceptor firing was ineffective in maintaining a hyperventilation The relatively smaller number of mitochondria in the central nervous system of the newborn (Samson Jacobs and Balfour 1960 Skoglund 1967) may indicate that central neurons including those concerned in respiratory control are more sensitive to changes in pO_2 variations which might change their threshold for stimulation (cf Skoglund 1967 c) The aerobic metabolism actually decreased during hypoxia since the arterial pCO_2 decreased simultaneously with decreasing ventilation This is in accordance with earlier findings (Adamssons 1959 Dawes and Mott 1959 Hill 1959) CO production also probably decreased during hypercapnia (Chapter VIII p 89) From this it might be suggested that the reason for the secondary ventilatory depression in response to CO_2 in the newborn is principally the same as that for the depression caused by hypoxia viz a decrease in aerobic metabolism with a conseq[illegible] change in central threshold This assumtion is supported by the apparent [illegible] tween the respiratory records illustrating the biphasic ventilatory [illegible] hypoxia and hypercapnia respectively (see Fig 24 p 57 and Fig [illegible] Further in both cases gasping sometimes occurred which might [illegible] center becomes unresponsive to the pulmonary tension recepto[illegible] Godfrey 1966) In adult animals also oxidative metabolism is depr[illegible] (see Holmdahl 1956) but much higher concentrations are neces[illegible] The finding that newborn animals are more susceptible to barb[illegible]

which also inhibits oxidative metabolism further supports the conclusion that the reason for the greater sensitivity of the newborn to hypoxia and hypercapnia is a general effect on the neurons due to a depression of their oxidative metabolism

It may be noted that in a few cases the newborn continued gasping for a considerable period of time in spite of a severe hypoxia It was suggested long ago that the newborn animal has a rhytmic primitive spinal respiratory center (see Langendorff 1881) and the possibility must be considered that the gasps are generated from such a center

In spite of the fact that the newborn can survive oxygen lack for a much longer time than adults (see Mott 1961) the respiratory activity is more sensitive to oxygen lack and ceases more rapidly The reason for the longer survival time is of course a much greater ability to utilise anaerobic energy production (see Himwich 1951)

In the General Introduction it was aksed whether the correlation between functional and morphological maturation such as has been found in different *somatic afferent systems is also the case for afferents of direct importance in* respiratory control It was found (Chapter V and VI) that the bronchial and sinus nerve afferents in contrast for example to sural nerve afferents were able to fire continually in response to a maintained stimulus in spite of the fact that they were of similar or even smaller diameter The paranodal $NADH_2$ reductase activity however exhibited a more mature pattern in the bronchial and sinus nerve fibres in relation to their size than in the sural fibres Thus as suggested by Berthold and Skoglund (1967) the stage of maturation of the node paranode region appears to be a better correlate to functional maturity than fibre size Since the reductase is mainly mitochondrion bound (De Duve Wattiaux and Baudhuin 1962) the finding supports the hypothesis that the paranodal mito chondria are of importance for continual impulse transmission in myelinated mammalian nerve fibres (Berthold and Skoglund 1967)

The increase in mitochondrion bound enzyme activity in the respiratory muscles (Chapter VII) is essentially analogous to the postnatal increase in the nerve fibres It may thus be postulated that postnatally there is an increasing ability of the respiratory muscles to perform maintained contractions In the newborn the respiratory frequency is higher than in the adult (p 22) and the duration of each inspiratory contraction thus shorter Part of the effects seen after vagotomy might in fact be attributed to an exhaustion of the respiratory muscles and not only of the respiratory neurons (cf p 103)

The results of the injections of acids and bases (Chapter VIII) need some comment since their interpretation may necessitate a reevaluation of the concept of central chemoreceptors Thus when injecting a base in the newborn quite unexpectedly an augmentation of ventilation with a consequent increased CO_2

elimination was often obtained Part of the explanation for this might be that the base improves the condition of the effector part of the respiratory system which may have been dep essed by the slight acidosis initially prevailing (most newborn animals were slightly acidotic at the on et of the experiments (see p 24) But the finding that the afferent dis harge increased in response to the injection of the base and that pain for example provoked by pinching the tail gave a similar respiratory reaction points to the possibility that an increased diffuse input is the cause of this ventilatory augmentation In general it might therefore be asked whether changes in acid base balance apart from affecting the respiratory neurons directly also changes the afferent input to them

In the newborn inhibition of spinal reflexes has been shown to be weak (Skoglund 1960 d Wilson 1962) and stimulation of the skin afferent input gives mainly excitat on (Ekholm 1967) The question might be raised as to whether the difference in response between the newborn and adult animal with regard to the change in ventilation caused by a change in acid base balance of the blood is a consequence of changes in the relative influence of excitatory and inhibitory stimuli (cf Ekholm 1967) upon the respiratory neurons The afferent input was found to increase also in older animals in response to the injection of a base but the effect at that stage of development was inhibition of respiration instead of facilitation as in the newborn It might be suggested that the reason for this is an increased effect of both peripheral and central inhibitory afferents on the respiratory neurons As pointed out earlier (p 14) it seems generally held that most excitable tissues are depressed by acidosis and excited by alkalosis but that the neurons of the reticular activating system react oppositely in this respect However this has only been shown indirectly (see Wyke 1963) and it might be suggested that the arousal reaction consequent to acidosis as well as the increased ventilation in the adult (see p 14) is caused by a reduction of the inhibition from other sources on the reticular activating system and not by a direct effect on the reticular neurons Part of the explanation for the changes taking place during development might be a change in the neuronal sensitivity in this respect

Finally some comments on the possible applicability of the present results to the newborn infant seem pertinent The fibre calibre spectra of lumbar spinal roots of newborn infants are the same as those obtained from kittens about 3 weeks old (Nystrom and Skoglund 1965) Moreover the ultrastructure of the node paranode region appears to be the same in nerve fibres of similar size from cats and man (Hildebrand and Skoglund 1967) Since the muscle spindles of three weeks old kittens are gamma" driven by analogy it might be inferred whether the gradually developing "gamma" control of the muscle spindles has not started in the human infant at birth Since however (a) the relative growth of small

fibres such as "gamma" efferents is slower than that of bigger fibres (Skoglund 1960 b) (b) the growth of nerve fibres is slower in man than in the cat (cf Rexed 1944 and Skoglund and Romero 1965) and (c) the thoracic region is more immature than the cervical and lumbar regions in the newborn infant (Rexed 1944) the question of whether the intercostal muscle spindles do have a "gamma" control needs further study A possible approach might be to investigate electromyographically the response of this musculature to added respiratory load The finding by Cross et al (Cross Klaus Tooley and Weisser 1960) that the "Hering Breuer inflation" reflex is stronger in the newborn might actually indicate a lack or a less developed "gamma control at this stage (see above p 104)

As pointed out earlier the newborn infant shows a compensated respiratory alkalosis some 24 hours after birth (see Purves 1966 c) It was found (Chapter VIII) that an injection of a very week acid into a newborn kitten (0 05 N HCl) sometimes gave an overcompansatory ventilatory response indicating a greater sensivity of the "central chemoreceptors In fact the compensated respiratory alkalosis may be a reflection of an increased sensitivity to the hydrogen ions The newborn infant also seems to be more easily depressed by acidosis (Bland 1956 Bucci Scalamandre Savignoni and Mendicini 1965) which points to a different sensitivity of the immature respiratory neurons which further indicates that the present results may be applicable to the newborn infant as is also the case with the results obtained in response to hypoxia The reason why the newborn infant seemingly regulates its respiration as in the adult (cf General Introduction) might be that different compensatory mechanisms which later become concealed (see above) are dominant as e g the paradoxical reflex of Head

Great caution must however be exercised when directly applying experimental results from a species to another The postnatal duration for example of the dominance of the "paradoxical reflex" of Head must be ascertained in order to evaluate the relative importance of the different reflex influences for the regulation of breathing and this requires more experimental work in both man and animals

General summary

The present work is an investigation of postnatal changes in respiratory regulation in cats and rabbits The aim was to study which of the respiratory control systems are already functional at birth or if as yet immature how they develop postnatally Both morphological and physiological methods have been used

Chapter I gives a brief review of the relevant literature Chapter II describes the methods used when recording respiration arterial pCO_2 PO_2 and pH and blood pressure continuously as well as the methods of anesthesia decerebration etc Chapter III gives an account of the postnatal changes in breathing frequency and its relation to anesthesia and decerebration This Chapter also gives blood acid base values in anesthetized and decerebrate animals of different ages used in the present work

Chapter IV describes the effect of section of the vagus and sinus nerves on respiration and arterial pH and pCO_2 It was found that vagotomy initially gave rise to essentially the same change in respiratory pattern in cats and rabbits of all ages from birth upwards viz a prolongation of the inspiratory phases In animals below 3 weeks however the consequent decrease in respiratory frequency was more pronounced than in animals above 4 weeks of age Moreover in decerebrate or very lightly anesthetized animals below 3 weeks respiration often became periodic or gasp like In both decerebrate and anesthetized animals of this age group ventilation gradually decreased with a consequent progressive accumulation of CO_2

If the sinus nerves were sectioned after vagotomy there was only a slight ventilatory decrease in animals above 4 weeks of age but a marked depression in the animals below 3 weeks of age were a complete respiratory failure often ensued within a short time

Chapter V describes experiments on the vagal pulmonary tension receptor afferents It was shown that the Hering Breuer inflation reflex is present in the newborn kitten Direct recordings from the vagal pulmonary tension receptor afferents showed that they fired with lower frequencies in the newborn than in the adult but that they were able to fire continually in response to a maintained stimulus This latter is in striking contrast to the hindlimb muscle articular and skin receptors which can only fire short bursts (see Skoglund 1960 c and Ekholm 1967) A comparison between the calibre spectrum of a bronchial nerve (which containes the afferents from the pulmonary tension receptors) and the sural nerve (containing the afferents from hindlimb skin receptors) made on gluturaldehyde fixed osmium stained material revealed that the size distribution of the fibres in these nerves were similar in the newborn Postnatally however the bronchial nerve fibres grow much less The paranodal $NADH_2$ reductase

activity was compared in the sural and bronchial nerve fibres The bronchial fibres showed a more mature pattern in relation to their size whereas only type 1 and 2 fibres (classification of Berthold and Skoglund 1967) could be identified in the sural nerve at birth type 3 fibres we e also present in the bronchial nerve of the same newborn animal

Chapter VI describes some aspects of the physiology and morphology of the peripheral chemoreceptor afferents Recordings from the carotid chemoreceptor afferents revealed that they fired continually during air breathing increased their discharge in response to administration of 10 % O_2 and decreased or ceased firing with pure oxygen The calibre spectrum of the sinus nerve revealed that the fibres were generally smaller than those of the sural and b onchial nerves at birth and that the postnatal growth was even less than that of the bronchial fibres Like the latter however sinus fibres showed a more mature NADH reductase pattern in relation to their size than the sural ones at birth

In Chapter VI the ventilatory reactions to changes in oxygen concentration of the respired air are also described Administration of pure oxygen caused a marked initial ventilatory diminution at all ages when the pheripheral chemo receptors were intact which means that these receptors continually facilitate re piration during air breathing in the newborn as in the adult After denervation of these receptors there was no change in cats and rabbits above 8 weeks of age but a progressive increase in ventilation in animals below 3 weeks old On administrat on of 10 % O_2 there was a biphasic ventilatory response in animals 4 weeks of age and less in spite of an increasing discharge from the peripheral chemoreceptors but a sustained hyperventilation in animals above 8 weeks of age After denervation there was in all animals below 4 weeks old and in deeply anesthetized animals above 8 weeks a rapid ventilatory depression whereas lightly anesthetized animals above 8 weeks were unaffected

In a few animals about 1 week old which were tested in this respect the ventilatory depression during hypoxia was accompanied by a decrease in arterial pCO_2 and increase in pH which indicates that the oxidative metabolism was reduced The consequent decrease in CO_2 stimulation might partly explain the secondary ventilatory depression although also the central threshold for the chemoreceptor discharge seemed to increase

The first part of Chapter VII deals with the importance of the vagal and thoracic dorsal root afferents for inspiratory intercostal muscle activity recorded as the electromyogram (EMG) Section of the vagus nerves produced a pro nounced increase in EMG activity at all ages Section of the thoracic dorsal roots did not affect the intercostal muscle activity in newborn animals but caused a considerable decrease in EMG in animals above 2 weeks old

Tracheal occlusion before inspiration caused a considerable increase in in

spiratory intercostal muscle activity at all ages when the vagi were intact Such a response was also present in animals above 4 weeks of age after vagotomy but absent in animals below 10—14 days of age After a subsequent section of the thoracic dorsal roots tracheal occlusion was without effect on intercostal muscle activity at all ages

The fibre calibre spectra of the 3rd and 6th thoracic dorsal and ventral roots and intercostal and phrenic nerves were also studied in Chapter VII Osmium stained paraffin embedded material was used It was found that the ventral roots and phrenic nerves were in a slightly more advanced state of development than the dorsal root fibres at birth which in turn were of the same size as the dorsal roots of the lumbar region as described by Skoglund and Romero (1965) A peak of "gamma" fibres could not be identified in animals below 25 days in the intercostal muscle nerves A peak of small fibres (about 2 μ) was present in the ventral roots but it was concluded that this mainly consisted of preganglionic sympathetic efferents

The results indicate that part of the reason for the negligible importance of the thoracic dorsal roots for respiratory control in the newborn is a lack of gamma control of the intercostal muscle spindles and a relative immaturity of their afferents

In Chapter VIII the ventilatory responses to administration of CO_2 in the inspired air and to infusions of acids and bases are described Administration of CO_2 caused a ventilatory augmentation before as well as shortly after denervation of the peripheral chemoreceptors which indicates that the central chemoreceptors are functionally active in the newborn However in response to 6 5 % CO_2 the ventilatory response was regularly biphasic and after an initial augmentation a secondary diminution occurred in animals below 2—3 weeks old Half an hour to an hour after denervation of the peripheral chemoreceptors no response or only a further ventilatory depression was seen on administration of CO_2

Injection of 1 0 ml/kg of a 0 5 N HCl or NH_4Cl solution stimulated respiration in animals of all ages but there was often an initial and sometimes a maintained respiratory depression in animals below 3 weeks of age After the initial diminution however respiration was usually stimulated A tenfold reduction in the dose of acid often caused overcompensation with hyperventilation and a consequent secondary decrease in pCO_2 and increase in pH

Injection of 1 0 ml/kg of a 1 0 M NA_2CO_3 solution caused a ventilatory diminution in animals above 4 weeks of age An equivalent dose of $NaHCO_3$ firstly stimulated ventilation slightly but then also inhibited it in this group of animals The effect of one or two successive injections was always the same

In animals below 3 weeks of age a first injection of the same relative dose of a base quite unexpectedly caused a paradoxical response in that ventilation

increased A second injection on the other hand regularly produced an adult response viz a ventilatory diminution

In Chapter VIII the effect on the activity in the vagus nerve afferents of injections of acids and bases is also studied Whereas an injection of an acid did not have any observed effect the first injection of a base always caused an increase in discharge in the vagal afferents at all ages In animals above 4 weeks also one or two successive injections caused an increased discharge whereas a second dose in a newborn animal caused only a transient initial increase followed by a secondary decrease in the discharge

Finally the blood carbonic anhydrase activity was estimated by a simple colorimetric method and found to increase postnatally The adult value was reached at about 4 weeks of age in kittens

In the General Discussion it was concluded that the respiratory control systems which are active in the adult are not fully developed in the newborn cat and rabbit the most important difference probably being the negligible influence of the thoracic dorsal root afferents in the newborn stage The results of the present work explain the deleterious effect of vagotomy in the newborn The relation between function and morphology of the developing nerve fibres was discussed and it appears that the stage of maturity of the node paranode region is a better correlate to functional maturity than fibre size The depressive effects of hypoxia and hypercapnia found here and the somewhat unexpected results of the injections of acids and bases in the newborn were discussed against the possibility of a reevaluation of the concept of the central chemoreceptors" Finally the possible applicability of the present findings to the newborn infant was discussed

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THE ADRENERGIC TRANSMITTER OF THE FEMALE REPRODUCTIVE TRACT DISTRIBUTION AND FUNCTIONAL CHANGES

BY

NILS OTTO SJÖBERG

LUND 1967

ACTA PHYSIOLOGICA SCANDINAVICA
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The present dissertation is based upon the following papers

1 OWMAN CH, and N O SJOBERG Adrenergic nerves in the female genital tract of the rabbit With remarks on cholinesterase containing structures *Z Zellforsch* 1966 *74* 182—197

2 OWMAN CH, E ROSENGREN and N O SJOBERG Origin of the adrenergic innervation to the female genital tract of the rabbit *Life Sci* 1966 *5* 1389—1396

3 ROSENGREN, E and N O SJOBERG The adrenergic nerve supply to the female reproductive tract of the cat *Amer J Anat* 1967 *121* 271—284

4 OWMAN CH, E ROSENGREN and N O SJOBERG Adrenergic innervation of the human female reproductive organs A histochemical and chemical investigation *Obstet and Gynec* 1967 *30* In press

5 OWMAN CH and N O SJOBERG Difference in rate of depletion and recovery of noradrenaline in short and long sympathetic nerves after reserpine treatment *Life Sci* 1967 In press

6 ROSENGREN E and N O SJOBERG Changes in the amount of adrenergic transmitter in the female genital tract of rabbit during pregnancy *Acta physiol scand* 1967 In press

7 SJOBERG N O, Considerations on the cause of disappearance of the adrenergic transmitter in uterine nerves during pregnancy *Acta physiol scand* 1967 In press

8 SJOBERG N O Increase in transmitter content of adrenergic nerves in the reproductive tract of female rabbits after oestrogen treatment *Acta Endocr* (Kbh) 1967 In press

They will be referred to below by PAPER 1 PAPER 2 *etc*

EXPERIMENTAL DESIGN

The basis of the investigation has been a study of the presence and cellular distribution of certain monoamines—dopamine adrenaline noradrenaline and 5 hydroxytryptamine—in the ovary oviduct uterus and vagina in various mammalian species under normal and certain experimental conditions The study was carried out on guinea pigs rabbits cats, and humans

1 Monoamine containing nerves and other cell systems were studied histo chemically with the fluorescence technique of Falck and Hillarp The monoamines visualized were identified and their concentrations in the tissues were determined by spectrofluorimetric methods
2 Among the monoamines analyzed noradrenaline was by far the dominating amine measured in the tissues and was present in systems of adrenergic neurons
3 The origin and organization of these systems were elucidated in denerva tion experiments These experiments showed that the smooth muscle coat of the reproductive tract received part of its adrenergic innervation by way of a special kind of neurons whose cell bodies like those of the postgang lionic parasympathetic neurons were situated near the effector organ This finding prompted pharmacological experiments to elucidate any functional difference between these so called short adrenergic neurons and ordinary long adrenergic neurons emanating from pre and paravertebral ganglia
4 Against the background of the finding that the female genital tract has a special organization of its sympathetic innervation studies were performed to ascertain any sign of functional involvement of these nerves during cer tain conditions such as pregnancy and hormonal treatment

MATERIAL, TECHNIQUES AND DRUGS

The material consisted of 244 rabbits, 29 cats, and 64 guinea pigs. In addition, tissue specimens were obtained from about 50 patients in association with operations. The reproductive organs were examined for certain monoamines (dopamine, noradrenaline, adrenaline, and 5-hydroxytryptamine) by a combination of fluorescence microscopic and spectrofluorimetric techniques.

Preparations used for fluorescence microscopy were quenched to the temperature of liquid nitrogen in a propane-propylene mixture and freeze-dried at −20° to −30° C. The specimens were then exposed to gaseous formaldehyde from paraformaldehyde (Merck, at +80° C for one hour (to demonstrate 5-hydroxytryptamine and primary catecholamines) or three hours (to obtain an optimal reaction of any secondary catecholamines, such as adrenaline when present in the tissues). Under the nearly dry conditions used the histochemical reaction between the amines and formaldehyde give rise to reaction products (fluorophores) emitting an intense fluorescence when activated by light of short wave length (Falck 1962, Falck, Hillarp, Thieme and Torp 1962, Corrodi and Hillarp 1963, 1964). In a microscope with an activation filter having a peak between 390 and 410 mμ (Schott BG 12) and a secondary filter with a high absorption below 490 mμ (Schott OG 4), the light emitted by the catecholamine fluorophores is green, whereas that of 5-hydroxytryptamine is yellow. Further details about the technical procedures have been given by Falck and Owman (1965). The chemical reactions and principles underlying the histochemical method have been extensively reviewed by Corrodi and Jonsson (1967).

Tissues used for spectrofluorimetric analysis were homogenized in perchloric acid at 0°–4° C. The content of dopamine, noradrenaline and adrenaline was measured according to Bertler, Carlsson, Rosengren and Waldeck (1958) as modified by Häggendal (1963). The amines in the tissues were identified by comparing the excitation and emission spectra with those of authentic monoamines in an Aminco-Bowman spectrophotofluorometer.

Cholinesterase activity was demonstrated in fresh cryostat sections (Paper 1) according to Koelle (1963) with the use of proper inhibitors for non-specific and specific cholinsterase (see Holmstedt 1957, Koelle 1963).

In some experiments the vascular system was demonstrated by injection of India Ink (see Papers 1, 3 and 7), after which the tissue specimens were prepared for fluorescence microscopy as above.

The origin of the sympathetic innervation of the genital tract was investigated in guinea pigs, rabbits, and cats by histochemical and chemical analysis of the disappearance of the adrenergic transmitter (see FALCK 1962) in denervation experiments which are described in detail in PAPERS 2, 3 and 7.

The drugs used in the investigation were in the following forms:

1. Nembutal (Mebumalnatrium, ACO) 6 % standard solution.
2. Urethan, 20 % solution in 0.9 % physiologic saline.
3. Reserpine (Serpasil Ciba), 2.5 mg/ml in standard solution.
4. 17 β estradiolbenzoate (Mann) 10 μg/ml in peanut oil solution.
5. India Ink in the commercial form diluted with an equal volume 0.9 % saline with the addition of a few drops of amyl nitrate and filtered before injection.
6. Drugs used in association with operations at which human material was obtained: Atropin (ACO) 0.1 mg/ml solution, Petidin (ACO) 50 mg/ml solution, Pentothalsodium (Abbott), 25 mg/ml solution, Celocurin (Vitrum), 50 mg/ml solution.

The Student's *t* test (BONNIER and TEDIN 1957) was used in the statistical evaluation of differences in the mean values of the catecholamine contents.

ORGANIZATION OF ADRENERGIC INNERVATION OF REPRODUCTIVE ORGANS IN NON-PREGNANT FEMALES

Review of literature

The first critical and most detailed work on the innervation of the internal female genital organs was presented by FRANKENHÄUSER (1867) whose comprehensive monograph was to form the basis of many later publications According to the classical work of Frankenhauser the human genital organs receive their motor innervation from the sympathetic hypogastric plexus and from the pelvic nerves the latter constituting the sacral parasympathetic system The hypogastric plexus is a continuation of the inferior mesenteric plexus which in turn arises from fibres in the aortic plexus and from the ganglia in the lumbar part of the sympathetic chain The pelvic nerves originate from the first three to four sacral nerves According to FRANKENHÄUSER (1867) these two sources converge into a ganglion which is located close to the uterine cervix and from which nerve fibres spread into the various genital organs The view that Frankenhausers ganglion consists mainly of one large ganglion formation has since been accepted by many workers in this field (HASHIMOTO 1904 JUNG 1905 GEMMELL 1926 KRANTZ 1959) On the other hand JASTREBOFF (1881) DAHL (1916) and DAVIS (1933) could only find extremely small ganglia in Frankenhausers ganglion formation and suggested that it should be regarded as a pure plexus formation

According to FRANKENHÄUSER (1867) the sympathetic contribution to the innervation of the female genital apparatus is excitatory while the parasympathetic nerves probably have an inhibitory effect on the motor activity of the uterus Especially concerning the sacral parasympathetic contribution by the pelvic nerves no general agreement has been achieved Some authors have thus reported contraction of the uterus on stimulation of the sacral nerves (KORNER 1865 ROHRIG 1879 BASCH and HOFMANN 1877 KELLNER 1887 WHITEHOUSE and FEATHERSTONE 1923) while others claim that the sacral nerves have an inhibitory effect on the uterus (e g DAHL 1916) LANGLEY and ANDERSON (1895 a) however were of the opinion that the sacral nerves do not partake in the motor activity of the uterus Their opinion appears to be the one most widely accepted

On the other hand there appears to be better agreement concerning the physiological importance of the sympathetic contribution via the hypogastric plexus The origin of the sympathetic nerves their course and distribution as described in detail by LANGLEY and ANDERSON (1895 a b 1896 a b) form the basis of most modern descriptions of these nerves These authors studied

the innervation of the genital organs in the cat and the rabbit in dissection studies in physiological experiments and by analyses of nerve degeneration phenomena after division of the sacral fibres LANGLEY and ANDERSON (1895 a) found the sympathetic nerve fibres of the genital organs to originate in the spinal cord from the third to fifth lumbar segment from which the nerves entered the fourth to sixth lumbar ganglia in the sympathetic chain From the sympathetic ganglia the fibres could be traced via the inferior mesenteric ganglia into the two hypogastric nerves Each of these two nerves divides into a dorsal and ventral branch The dorsal branch runs to the pelvic plexus which is identical with Frankenhausers plexus where it meets the parasympathetic fibres which according to the authors originate from the first three to four sacral roots The ventral branch on the other hand runs to the wall of the vagina to join ganglion formations which are situated in the dorso lateral part of the vaginal wall (LANGLEY and ANDERSON 1896 b) This organization of the sympathetic innervation has since been confirmed by DAVIS (1933), REYNOLDS and KAMINESTER (1935 a) and REYNOLDS (1949)

LANGLEY and ANDERSON (1895 a) stimulated the lumbar nerves lumbar ganglia inferior mesenteric ganglia and hypogastric nerves electrically and invariably noticed that it produced muscular contraction and vasoconstriction of the vagina uterus and oviduct The authors also gave large doses of the ganglionic blocking agent nicotine (LANGLEY and ANDERSON 1895 b) after this pretreatment electrical stimulation of the nerves in the above mentioned regions produced no effect The authors therefore concluded that the fibres in the hypogastric nerves were partly preganglionic probably forming synapses in sympathetic ganglia situated peripherally to the inferior mesenteric ganglia i e near the effector organ Stimulation of the sacral nerves on the other hand had no effect on the contractions of the uterus (LANGLEY and ANDERSON 1895 a) a result later confirmed by DALE and LAIDLAW (1912) Furthermore sectioning of these nerves did not result in degeneration of the terminal network in the genital organs (LANGLEY and ANDERSON 1896 a) It was therefore concluded that the sacral nerves did not contribute with any motor fibres to the internal female genital organs

It is evident that already LANGLEY and ANDERSONs (1895 a b 1896 a b) publications gave a detailed description of the origin course and distribution of the nerves to the female genital tract The discussion of the arrangement of the autonomic innervation of the genital organs has nevertheless continued especially concerning the uterus because this is often used as a model organ in the pharmacological studies of certain autonomic mechanisms Thus, after administration of a large dose of nicotine CUSHNY (1906) stimulated the hypogastric nerves without obtaining any response of the uterus and therefore like LANGLEY and ANDERSON (1895 b) assumed that the sympathetic nerves to the genital organs had a more peripheral origin This assumption has however not been generally accepted. According to SCHOFIELD (1952) the hypogastric nerves contain postganglionic fibres since the response to stimulation of

these nerves was not inhibited by ganglionic blocking agents, such as nicotine or hexamethonium It should be pointed out that similar results have been obtained with hexamethonium in studies on the internal genital organs of male animals after stimulation of the hypogastric nerves (BURNSTOCK and HOLMAN 1960 RAND 1961) With the present knowledge that the male genital organs receive their sympathetic innervation from a peripheral formation of ganglia (SJOSTRAND 1965) it is obvious that results obtained in experiments with hexamethonium may be misleading in cases when the drug is used in insufficient amounts

KURDINOWSKY (1905) CUSHNY (1906) and DALE (1906) were among the first to find adrenaline to have the same effect on the motility of the uterus as stimulation of the hypogastric nerves Other investigators (KEHRER 1907 ADLER 1912, GUNN and GUNN 1914) on the other hand found that adrenaline sometimes had a relaxing effect on the uterus GRUBER (1933), who believed the hypogastric nerves to possess both excitatory and inhibitory fibres suggested that such discrepancies might be due to the relative amount of these two types of fibres present in the species studied or to the hormonal status of the animal

The question of the autonomic innervation obtained a new aspect through DALE s (1933) work demonstrating that adrenaline or an adrenaline like substance mediates the effect of sympathetic nerve endings onto the effector cells, while the parasympathetic nerves act by way of acetylcholine BACQ and FISCHER (1947) were among the first to suggest that the mediator in the cat uterus might be noradrenaline instead of adrenaline EULER (1946) demonstrated the presence of noradrenaline in the uterus i e the substance that proved to be the adrenergic transmitter in the sympathetic nervous system (see EULER 1956 1961) In the light of the fact that noradrenaline is the transmitter substance in peripheral sympathetic nerves, GREEFF and HOLTZ (1951) studied the effect of noradrenaline on the uterus and obtained a more uniform excitatory response of the uterus in various species in contrast to what had been found previously with adrenaline Moreover after stimulation of the hypogastric nerves MANN and WEST (1951) and VOGT (1965) found the amount of noradrenaline in the ovarian venous blood to be several times that of adrenaline The demonstration of large amounts of noradrenaline in the hypogastric nerve trunk has led to the conclusion that this is mainly an adrenergic nerve (VARAGIC 1956 VANOV and VOGT 1963)

Of the internal genital organs in various mammals including man (see KRANTZ 1959) the uterus has attracted most interest The innervation of the ovary oviduct and vagina has received much less attention According to the literature, the ovary is innervated partly from the ovarian plexus receiving fibres from the renal plexus and partly by the hypogastric nerves (DAHL 1916 KRAUL 1927 STOHR 1928 MITCHELL 1938 WATZKA 1957) The autonomic nerves to the oviduct run both via the ovarian plexus and Frankenhauser s plexus (STOHR 1928 HORSTMANN and STEGNER 1966) LANGLEY and

ANDERSON (1895 a) suggested on the basis of studies on cats and rabbits that the entire sympathetic innervation reaches the oviduct by way of the hypogastric nerves This was confirmed by BRUNDIN (1965), who furthermore suggested on the basis of denervation experiments that this was not the only source of sympathetic innervation

LANGLEY and ANDERSON (1895 a) showed that also the vagina is innervated by sympathetic fibres from lumbar nerves via the hypogastric nerves a finding that is now generally accepted Accordingly, stimulation of the hypogastric nerves resulted in muscle contraction and vasoconstriction of the vagina in the same way as in the uterus and the oviduct The findings of LANGLEY and ANDERSON (1895 a) regarding the vaginal sympathetic innervation has since been confirmed by STARLING (1900), GUNN and DAVIES (1920) and GUNN and FRANKLIN (1922)

The above survey shows that as early as 1895 it was suggested that at least part of the sympathetic postganglionic nerves to the female genital tract originated from ganglia situated in the vicinity of the effector organ i e with principally the same arrangement as that of the postganglionic parasympathetic fibres Yet this conception has not been generally accepted during the following 70 years research into the sympathetic innervation of the female reproductive organs Furthermore the intrinsic nerve supply to the female genital organs has been the subject of numerous investigations Though these studies using the classical histological methods have contributed considerably to our knowledge of the morphology and topography of the nerve fibres they have not been able to differentiate with certainty between adrenergic and other types of nerve fibres This must be ascribed to the lack of specific methods allowing complete and detailed information as to the organization of the sympathetic or adrenergic innervation of these organs

Terminal adrenergic innervation in female genital tract

(PAPERS 1 3 4 and 7)

Fluorescence microscopy of the female reproductive tract (ovary oviduct, uterus and vagina) revealed a system of green fluorescent adrenergic nerves varying in density from organ to organ The fluorescent nerve fibres proved to be distributed in principally the same way in all the species investigated (guinea pig rabbit cat and man) Prolonged treatment with formaldehyde in attempts to visualize adrenaline (FALCK HÄGGENDAL and OWMAN 1963) did not result in the appearance of any further green fluorescence in any of the regions in the species studied Accordingly spectrofluorimetric determinations of catecholamines in the different organs revealed significant levels of nor

adrenaline only The number and the fluorescent intensity of the adrenergic nerves turned out to be well correlated with the levels of noradrenaline measured in the respective organs

Thick bundles of preterminal nerve fibres of smooth appearance and emitting but weak fluorescence entered the *ovary* along blood vessels in the hilar region Within the ovarian stroma the bundles split up into a large number of green fluorescent nerve terminals running alone or forming small bundles which coursed in all directions The density of the nerve plexuses was particularly pronounced in the cat (PAPER 3), in man (PAPER 4) and in the dog (OWMAN and SJOBERG 1967 b) In experiments in which the vascular bed had been demonstrated bv injection of India Ink, it could be clearly shown that a considerable portion of the vascular system was supplied by adrenergic fibres forming wide meshed networks However part of the ovarian adrenergic nerve terminals were of non vascular nature A substantial number of these terminals were often seen to run for example in close contact with follicles in various stages of development

The density of the ovarian adrenergic innervation agreed well with the amount of noradrenaline present in the various species The noradrenaline concentration in the ovary of cat (4 93 μg/g) and man (0 86 μg/g) was higher than in the other reproductive organs in these two species

Also the *oviduct* (Fallopian tube) had a fairly rich supply of adrenergic nerves Systematic analysis of serially sectioned oviducts revealed that part of the nerves ran along vessels in the entire organ, whereas the rest were distributed in smooth muscles and varied characteristically in number along the course of the oviduct In the ampulla onlv a few terminals occurred in the smooth muscles whereas most of the adrenergic nerves were exclusively vascular In a small region at the junction between the ampullary and the isthmic portion there was a marked and abrupt increase in the density of adrenergic muscular innervation (PAPERS 1 3 and 4) The increase was confined mainly to the prominent circular muscle layer The amount of nerves in this layer decreased slightly towards the uterus The adrenergic terminals usually ran parallel to and in close contact with, the muscle fibres in a way suggesting a true innervation This arrangement of the muscle terminals is similar to that found in other smooth muscles *e g* in the vas deferen (FALCK, OWMAN and SJOSTRAND 1965) where RICHARDSON (1962) in electron microscopic studies claimed the existence of a true synaptic contact between the nerve terminals and the smooth muscle cells

The above mentioned innervation pattern of the oviduct was principally identical in all species studied although it was sometimes possible to recognize an enhancement in the muscular innervation also in the tubo uterine junction of the cat (PAPER 3) Spectrofluorimetric determinations of the ampulla isthmus and intramural portion in man revealed a significantly higher concentration of noradrenaline in the isthmus (PAPER 4) In this calculation the amine concentration in each isthmus was expressed as the percentage of the

concentration of on one hand the ampulla and on the other hand the intra mural portion from the same patient This procedure minimizes the influence due to differences in concentration between individual patients

The conspicuously rich adrenergic nerve supply to the circular muscle layer of the isthmus confirms the findings by earlier investigations (BRUNDIN and WIRSEN 1964 a b BRUNDIN 1965) The increase of the thickness of the muscle wall together with the increasing number of nerves indicate a specialized function of this part of the oviduct in *e g* the transport of the ovum This assumption has found further support in experimental investigations in rabbits in which an adrenergic sphincter appears to operate at the isthmic level (BRUNDIN 1965) The isthmus of the rabbit Fallopian tube serves as an adre nergic sphincter closing the isthmic lumen The closing mechanism is obvious in non mated rabbits and it can be strengthened by stimulation of the hypo gastric nerve In mated animals the closure is less marked and the response to stimulation of the hypogastric nerve and to exogenous noradrenaline is weaker during the period of passage of the fertilized ova through the isthmus

In view of the present finding that the adrenergic innervation was parti cularly rich in the circular layer of the isthmic region adjacent to the ampulla it is probable that such a sphincter mechanism may be limited to this site rather than including the entire isthmus On the other hand there is no evidence for the belief that such a sphincter mechanism should operate also at a tubo uterine level at least not in rabbit or in man (see further BLACK and ASDELL 1958 GREENWALD 1963 RAURAMO SOILA GRONROOS and WEGE LIUS 1964)

In the rabbit the number of acethylcholinesterase-containing nerve fibres was small compared with the rich adrenergic innervation of the isthmus (PAPER 1) According to the hypothesis of Burn and Rand (see BURN 1963) acethylcholine might have a mediating role in the liberation of noradrenaline at terminals of adrenergic axons This liberation might be brought about by acetylcholine first being released by stimulation of the postganglionic neuron and then initiating or facilitating the release of noradrenaline either in the same nerve fibre or in an adrenergic terminal adjacent to the cholinergic fibre (JACOBOWITZ and KOELLE 1965) Such an interrelation between adrenergic and cholinergic mechanisms appears hardly likely in the rabbit's isthmus owing to lack of structural prerequirements the acetylcholinesterase containing fibres there being extremely few compared with the noradrenaline nerves

The muscular coat of the *uterus* in the animals examined consists of an outer longitudinal and an inner circular smooth muscle layer separated by a well developed vascular plexus Many of these vessels were enclosed by modera tely dense plexuses of green fluorescent varicose nerves Especially in prepara tions taken from animals examined after intravenous injection of India Ink it was possible clearly to distinguish the vascular innervation from fluorescent varicose terminals of non vascular nature which were fairly abundant in the myometrium Muscular nerves occurred both in the inner circular and in the

outer longitudinal smooth muscle layers and usually ran in close connection with the muscular fibres in a fashion similar to that found in the oviduct

The muscular adrenergic innervation of the cat uterus (PAPER 3) was richer than that in the rabbit (PAPER 1) and guinea pig (PAPER 7), which is reflected by the four to five fold higher noradrenaline content in the cat uterus (2 65 µg/pair)

Only in humans was it possible to demonstrate any regional variation in density of the uterine adrenergic innervation (PAPER 4) Thus, in the uterine fundus and corpus, the number of adrenergic nerve terminals was relatively smaller than in the cervix where the concentration of noradrenaline was about three times as high as in either the fundus or corpus This result was calculated by the Student s *t* test disregarding any correlation that could occur between the spectrofluorimetric values estimated within the same individual This means that when a positive correlation exists the significance reported in PAPER 5 is even somewhat too low

It is notable that intramural myomas were found to be completely devoid of adrenergic fibres and to contain no noradrenaline despite a well developed vascular supply (PAPER 4)

The uterus is a classical organ for pharmacological studies of certain adrenergic mechanisms *There is a considerable species difference in the amount of* catecholamines in this organ It is of obvious interest that it is now possible to show the cellular localization of uterine catecholamines An adrenergic innervation to both the vessels and the smooth muscle coat has regularly been demonstrated in the uterus of guinea pig rabbit and cat (PAPER 1 3 and 7) whereas in contrast to this the rat uterus only receives adrenergic innervation to its vascular system (NORBERG and FREDRICSSON 1966, OWMAN and SJOBERG 1967 b) Such a conspicuous species variation in the organization of the terminal autonomic innervation apparatus has been demonstrated elsewhere (cf EHINGER 1966 OWMAN and SJOSTRAND 1966 OWMAN 1967) and should apparently be taken into acount in functional studies on amine mechanisms in many organs The histochemical findings in the present human material indicate that a low concentration of noradrenaline does not necessarily mean the absence of a possibly important functional adrenergic innervation It was interesting to note that the concentration of noradrenaline in the human cervix was three times as high as elsewhere in the uterus (PAPER 4)

Also the *vagina* had a rich adrenergic nerve supply in all the species studied Throughout the length of the organ, intensely green fluorescent varicose fibres formed a dense three dimensional plexus including the smooth muscles and the vascular system The fibres coursed in all directions many lying in intimate contact with the muscle fibres In several instances varicose terminals were seen also in the mucosa but they always accompanied vessels and were never seen to enter the epithelium

Origin of the adrenergic innervation
(PAPERS 2, 3 and 7)

As mentioned in the review of the literature the sources of the sympathetic innervation to the female genital tract has been the subject of much controversy Though already LANGLEY and ANDERSON (1895 b) suggested that at least part of the sympathetic nerves to the female genital tract relay in a system of peripheral ganglia located in the vicinity of the effector organs the general opinion has been that the hypogastric nerves contain predominantly postganglionic fibres to the female genital tract This assumption is based mainly upon the finding that ganglionic blocking agents, such as nicotine and hexamethonium do not inhibit the response of the smooth muscles of the uterus to stimulation of the hypogastric nerves (SCHOFIELD 1952) On the other hand division of the hypogastric nerves at the level of the inferior mesenteric ganglia results in disappearance of only part of the noradrenaline in the rabbit oviduct (BRUNDIN 1965), and thereby suggests the existence of a sympathetic relay peripherally to the inferior mesenteric ganglia Moreover it has recently been shown that the smooth muscle coats of the internal genitals in the male are richly innervated by adrenergic terminals which do not reach the organs via the hypogastric nerves but originate exclusively in peripheral sympathetic ganglia located in the vicinity of the effector organs (FALCK OWMAN and SJOSTRAND 1965 OWMAN and SJOSTRAND 1965 SJOSTRAND 1965)

In the present investigation adrenergic ganglion formations were demonstrated at the utero vaginal junction and in the vicinity of the upper portion of the vaginal wall of all species studied (PAPERS 1 3 and 7) including man (PAPER 4) The ganglia contained both non fluorescent cell bodies and cells with a distinct green cytoplasmic fluorescence of moderate intensity All cells were of rather uniform size and both fluorescent and non fluorescent cells were enclosed by a number of adrenergic varicose terminals in a way suggesting a synaptic arrangement In a corresponding type of peripheral ganglion formations located intramurally in the trigonum region of the urinary bladder HAMBERGER and NORBERG (1965 a) produced evidence that the non fluorescent cells are cholinergic In sections cut at favourable planes preterminal fibre bundles could be seen to leave the ganglion formations Clusters of fluorescent nerve cells were found not only in the connective tissue immediately outside the cervix and the upper portion of vagina but also scattered beneath the surface of the vaginal wall in the guinea pig (PAPER 7) rabbit (PAPER 1) and cat (PAPER 3) Careful examination failed to reveal any formations of adrenergic ganglia in or near other parts of the reproductive tract

In order to ascertain whether the utero-vaginal ganglionic formations represented the above mentioned peripheral synaptic relay various denervation experiments were performed and followed at least one week later by histochemical and fluorimetric analyses of changes in the noradrenaline level in the genital organs of the rabbit cat and guinea pig This procedure is based on

the well known facts that the morphology the transmitter content and therefore the fluorescence of the postganglionic axons are not affected by severance of the preganglionic nerves (REHN 1958, FALCK 1962) On the other hand after excision of the postsynaptic nerve cells or division of the postganglionic axon there is a rapid loss of transmitter in the peripheral stump already within 15 hours During the following few hours the fluorescence becomes considerably weaker and by 48 hours after the operation it has disappeared from all the nerves (FALCK 1962) This rapid disappearance of the monoamine thus precedes the structural disintegration of the sympathetic fibres (HÅKANSON and OWMAN 1965)

After removal the L—S ganglia of the sympathetic chain the fluorescence of all ovarian nerves was abolished (PAPER 2) Most of the fluorescent vascular nerves in the oviduct uterus and vagina also disappeared (SJÖBERG 1967 c) The muscular innervation of the above mentioned organs was not overtly affected by this operation (PAPER 2)

After division of the hypogastric nerves (with or without excision of the inferior mesenteric ganglion formations) a slight reduction was often found in the number of nerves running in the muscle coat of the oviduct Spectrofluorimetricallly the operation caused a decrease of about 50 percent in the noradrenaline content of the rabbit oviduct (cf BRUNDIN 1965) (PAPER 2) In the cat there was no definite reduction in the total amount of nerves and accordingly the noradrenaline content remained unchanged (PAPER 3) The hypogastric nerve is thus responsible for at most half (depending on the species studied) of the adrenergic muscular innervation of the oviduct the rest being supplied from a more peripheral source The results of histochemical and spectrofluorimetric analyses of the uterus and vagina after this operation indicate that these organs receive at least half (in the cat PAPER 3) or none (in the rabbit PAPER 2) of their adrenergic innervation from the hypogastric nerves The situation in the uterus of the guinea pig (PAPER 7) is similar to that in the uterus of the rabbit

These results then do not support the conclusion by SCHOFIELD (1952) and by MILLER and MARSHALL (1965) that the hypogastric nerves in the rabbit contain mainly post ganglionic adrenergic fibres to the uterus the inability to modify the uterine response to hypogastric nerve stimulation in the presence of hexamethonium (SCHOFIELD 1952) might well have been due to too low a concentration of the drug This may be analogous to corresponding discrepances in studies on the vas deferens of the guinea pig this structure too receives its adrenergic innervation from peripheral adrenergic ganglia (SJÖSTRAND 1962 a b FALCK OWMAN and SJÖSTRAND 1965) but nevertheless it has been claimed (BURNSTOCK and HOLMAN 1960 RAND 1961) that the ganglionic blocking agent hexamethonium does not inhibit the motor response of the vas deferens to hypogastric nerve stimulation

The results of operations on the adrenergic ganglion formations at the utero vaginal junction (i e removal of visible ganglia in this region or transection

of the reproductive tract in a plane immediately above the ganglion formations) have lent strong support to the assumption that these peripheral adrenergic ganglia contribute to most if not all of that part of the adrenergic innervation not arising from pre and paravertebral ganglia (PAPERS 2 and 3) The fluorescence microscopic picture and the results of certain denervation experiments have shown that the terminal network in the vagina uterus and oviduct arises from large bundles of preterminal fibres which in serial sections can be seen to run parallel to the organs right from the vagina (PAPERS 1 3 and 7) This arrangement explains the finding of REYNOLDS and KAMINESTER (1935 b) that hypogastric nerve stimulation did not produce any uterine response when the parametrium at the uteri vaginal junction had been previously excised The sympathetic innervation of the ovary however, seems to be supplied by adrenergic fibres running along the ovarian artery (PAPER 3)

The results of the denervation experiments show that only *part* of the hypogastric nerves consists of postganglionic adrenergic fibres to the smooth muscles of the oviduct uterus and vagina whereas *most* of the sympathetic innervation of these structures arises from a more peripheral source i e by way of a neuron type which has been designated *short adrenergic neurons* (OWMAN and SJOSTRAND 1965) This special type of short adrenergic neurons distributed in a fashion resembling that of the parasympathetic postganglionic neurons has so far been found to occur only in the urogenital tract including the internal male genital organs (FALCK OWMAN and SJOSTRAND 1965 OWMAN and SJÖSTRAND 1965 SJOSTRAND 1965) urinary bladder (HAMBERGER and NORBERG 1965 a b) and urethra (OWMAN OWMAN and SJOBERG 1967) On the other hand the vascular nerves to the genital tract (and also non vascular nerves to the ovary) arise in the sympathetic chain It is notable that already LANGLEY and ANDERSON (1895 b) found that hypogastric nerve stimulation after ganglionic blockade with nicotine failed to produce any contraction of the uterus although a distinct pallor of the organ resulting from vasoconstriction could still be elicited by such stimulation

On the basis of the finding that the female genital tract in the experimental animals studied is supplied with short adrenergic neurons originating in the vicinity of the utero vaginal junction it is conceivable that the adrenergic ganglia found at this site represent a corresponding relay also in humans (PAPER 4)

Functional difference between "short" and "long" adrenergic neurons

(PAPER 5)

The short adrenergic neurons are of particular interest not because they differ in length from ordinary long sympathetic neurons but because they have proved functionally to constitute a special type of neurons

Thus in incubation studies on isolated noradrenaline granules obtained from

the vas deferens and the vesicular gland of bulls, LULIR and LISHAJKO (1966) and STJÄRNE and LISHAJKO (1966) found that the spontaneous release of noradrenaline was slower than in the corresponding granular fraction from the splenic nerve In accordance with this it has been shown that the vas deferens and vesicular gland (like the prostate) in the bull have a very rich adrenergic innervation by way of short adrenergic neurons (OWMAN and SJOSTRAND 1966) which was found to be the only noradrenaline stores occurring in large amount in the organ It was furthermore observed by NILSSON (1964) that after a single dose of reserpine, which is known to result in depletion of amines from the neuronal stores (see CARLSSON 1966) the adrenergic transmitter in the uterine sympathetic nerves of the rabbit disappeared at a substantially lower rate than in various other sympathetically innervated tissues This finding seems to be of particular interest in the light of the fact that the rabbit uterus (PAPER 2) has now been found to receive its sympathetic innervation by way of the special type of 'short adrenergic neurons

The reaction of the adrenergic transmitter to reserpine was therefore studied fluorescence microscopically in rabbits with the direct aim of comparing organs definitely innervated by long (heart and ovary) or 'short (uterus and vagina) adrenergic neurons The primary aim was the qualitative determination of the stage at which the fluorescence in the nerves had disappeared or started to return in the nerve terminals after reserpinization When comparing these results with fluorimetric determinations of the variation in the cardiac noradrenaline after the same type of reserpine experiments (CARLSSON ROSENGREN BERTLER and NILSSON 1957) it can be calculated that the histochemical technique used allows detection of noradrenaline down to about 5 per cent of the total concentration Moreover since the fluorescence was studied at close intervals after the injection of reserpine and since the organs innervated by either type of neurons were taken from one and the same animal the histochemical method used also allowed a semiquantitative estimation of the changes in the number of fluorescent nerves and their transmitter content (*i e* fluorescence intensity)

The experiments revealed that whereas the transmitter disappeared from the long neurons within 4 hours after a single i v injection of reserpine the rate of disappearance was considerably lower in the short adrenergic neurons in which the transmitter could be histochemically demonstrated even up to 30 hours after the injection A corresponding difference in the rate of depletion caused by reserpine has recently been found in a quantitative spectrofluorimetric comparison between the noradrenaline content of the male genital organs which are supplied by short adrenergic neurons and the submaxillary gland and heart (SJOSTRAND and SWEDIN 1967)

One reasonable interpretation seems to be that the rate of noradrenaline release also *in vivo* is lower in the short than in the long neurons This assumption is supported by the finding that the rate of depletion of the transmitter in the nerve terminals in the reserpinized animals was decreased by

severance of the preterminal trunk and increased by stimulation of the hypo gastric nerves

The noradrenaline fluorescence in the adrenergic nerve terminals reappeared almost one day earlier and recovered more rapidly, in the system belonging to the short neurons than in that belonging to the long neurons This may reflect a difference in the length of the axons in agreement with the view that the formation of the amine storage mechanism is initiated at the level of the nerve cell body (DAHLSTROM 1967) However, it cannot be excluded that the results reflect a *further* functional difference between the two types of neurons without any relation to their difference in length

NON-NEURONAL MONOAMINE CELLS IN FEMALE GENITAL TRACT

(PAPERS 1, 3, and 7)

Apart from the adrenergic nerves and ganglion formations, two further monoamine containing cell systems were demonstrated in the female genital tract namely chromaffin cells and vaginal epithelial cells containing 5 hydroxytryptamine

Clusters of small, branching cells exhibiting an intense green to yellowish catecholamine fluorescence were sometimes found in the vagina, close to the peripheral adrenergic ganglia (PAPERS 1 3 and 7) Similar cells have repeatedly been observed in relation to other nervous structures in many species (NORBERG and HAMBERGER 1964 OWMAN and SJOSTRAND 1965, NORBERG RITZEN and UNGERSTEDT 1966) The cells are probably related to previously described cells of similar morphology and known as 'chromaffin cells' because owing to their high amine content they usually stain with the chromaffin reaction (see LEMPINEN 1964) They are usually seen to give off a coarse process which can even be seen to run for a short distance inside preterminal bundles (see also OWMAN and SJOSTRAND 1966) However the total amount of chromaffin cells in the genital tract is probably too low overtly to influence the determinations of catecholamines in the genital organs

In the vestibular epithelium of the cat and rabbit vagina distinctly yellow fluorescent flask shaped cells were found (PAPERS 1 and 3) The formaldehyde induced fluorescence was characteristic of certain indole derivatives (FALCK 1962) In spectrofluorimetric determinations FORSBERG ROSENGREN and SJÖBERG (1964) found the presence of 5 hydroxytryptamine in the vestibular epithelium obtained by curettage of the rabbit vagina This finding provides further strong evidence that the yellow fluorescence in the flask shaped cells represents 5 hydroxytryptamine

FUNCTIONAL CHANGES IN NEURONAL TRANSMITTER CONTENT OF FEMALE GENITAL TRACT

(PAPERS 6 7 and 8)

During the last few years much interest has been devoted to alterations occurring in uterine adrenaline levels under the influence of female sex hormones as well as during pregnancy In the rat uterus the adrenaline content has been found to increase during the estrus phase and after estrogen administration (RUDZIK and MILLER 1962 WURTMAN CHU and AXELROD 1963 WURTMAN AXELROD and POTTER 1964 CHA LEE RUDZIK and MILLER 1965) The rat uterine adrenaline increases also during pregnancy (WURTMAN AXELROD and KOPIN 1963 CHA LEE RUDZIK and MILLER 1965) the increase being followed by a decrease *post partum* These changes however, seem to be of little relevance to the present investigation because the terminal adrenergic innervation in the rat differs in its principal arrangement from that in the other species presently studied including man in that practically all the nerves run in relation to the vessels (NORBERG and FREDRICSSON 1966 OWMAN and SJOBERG 1967 b) Moreover it has been questioned whether adrenaline has at all a neuronal localization in the uterus of the rat (WURTMAN AXELROD and KOPIN 1963 WURTMAN CHU and AXELROD 1963 WURTMAN AXELROD and POTTER 1964)

The now detailed knowledge of the adrenergic innervation pattern and the availability of both histochemical and chemical methods for analysis of the adrenergic transmitter have provided new possibilities for studying the female genital tract for changes in the noradrenaline content at a cellular level during pregnancy and after certain hormonal stimuli

Changes during pregnancy

(PAPERS 6 and 7)

In a combined histochemical and chemical investigation changes in the transmitter content of various parts for the female genital tract of the rabbit have been studied during three different stages covering the entire period of pregnancy (PAPER 6) Since the organs increase so much in weight during pregnancy the results have been expressed as changes in the *total* amine content of the organs By comparing the innervation pattern with changes in organ weight it has been possible also to assess changes in the true degree of innervation in the various organs

In spite of a considerable increase in the weight of the *ovary* during the first two weeks of pregnancy after which the weight persisted at a high level the total amount of noradrenaline was unchanged compared with that in non pregnant animals The arrangement of the nerves their morphology and transmitter content were essentially unchanged during pregnancy which was expected since the weight increase of the organ is due essentially to isolated changes *e g* development of corpora lutea

Also the *oviduct* increased markedly in weight during pregnancy The increase was accompanied by a rise in the total level of noradrenaline Fluorescence microscopy did not reveal any particular alteration in the arrangement of the sympathetic ground plexus or its transmitter content throughout pregnancy The number of fluorescent nerve terminals per amount of smooth muscle tissue remains unchanged This would mean that the total number of fluorescent nerve terminals increases in the rabbit oviduct during pregnancy

In the *uterus* during the first two weeks of pregnancy, the noradrenaline concentration when expressed *per unit of weight* was significantly lower than in non pregnant animals a finding in agreement with the calculation of CHA LEE RUDZIK and MILLER (1965) In the fluorescence microscope this manifests itself as a reduction in the *density* of adrenergic innervation, *i e*, increase in the amount of muscle tissue in relation to the number of nerve terminals However, a marked increase in the *total* noradrenaline content (and total number of fluorescent nerve terminals) was nevertheless observed but this increase was relatively smaller than the increase in the weight of the muscle In spite of a steady increase in the uterine weight there was a progressive reduction in the total noradrenaline content from the third week of pregnancy then reaching levels below those found in non pregnant animals In fact during the last period of pregnancy only few if any fluorescent nerves could be visualized in any part of uterus except in the cervix containing a substantial number of adrenergic nerve terminals with a reduced transmitter content the number was however much smaller than in non pregnant animals

In rabbit *vagina* a similar pattern of concomitant changes in the organ weight noradrenaline content and density of adrenergic innervation was also found although these changes were less conspicuous than in the uterus Thus a certain number of adrenergic nerves with a reduced fluorescence intensity persisted in the vagina even during the latter part of pregnancy

The experiments thus show that except in the ovary the total noradrenaline content and the total number of fluorescent nerves increase during the first half of pregnancy This does not necessarily mean the formation of new varicose axons but may well be explained by an enhanced fluorescence intensity in a population of nerve terminals having a transmitter content too low for histochemical detection in non pregnant animals An increase in the fluorescence intensity indicating an increase in the transmitter content of the fluores

cent varicose terminals visible already before pregnancy could not be established with certainty

During the latter half of gestation there was a marked *decrease* in the noradrenaline content of the vagina and particularly in the uterus where hardly any adrenergic nerves could be detected histochemically during the last days of pregnancy Flourescence microscopy indicated that the reduction is an effect of a decrease in the transmitter content of the individual axons finally producing especially in the uterus a dramatic decrease in the number of nerves visible because of their formaldehyde induced fluorescence It is possible that the marked decrease of the content of noradrenaline registered in the uterus during the latter part of pregnancy is simply the result of some mechanical interference with the network of nerves caused by the marked hypertrophy and distension of the muscle wall of these organs during pregnancy To check the validity of such an assumption is was desired to exclude these mechanical factors as far as possible Animals pregnant with fetuses in only one of the horns would serve this purpose well Guinea pigs were selected because they satisfy this demand and secondly because the organization of the adrenergic innervation in the uterus (PAPER 7) proved comparable to that in the rabbit (PAPER 1)

During the latter part of pregnancy the number of fluorescent nerves in the guinea pig uterus was markedly decreased in agreement with a pronounced reduction in the noradrenaline content (PAPER 7) These dramatic changes in the amount of uterine adrenergic nerves and noradrenaline found during pregnancy occurred also in the contralateral uterine horn containing no fetuses Since the uterine horn containing fetuses is distended and much larger than the contralateral horn without fetuses and since the noradrenaline content was markedly reduced in *both* uterine horns during pregnancy it seems hardly likely that a simple mechanical factor should be responsible for the disappearance of adrenergic transmitter during pregnancy The findings rather favour the possibility that the neurons have been affected by some hitherto unknown probably humoral factor

Influence of estrogen treatment

(PAPER 8)

In an attempt to establish whether a hormonal factor was responsible for any of the changes observed in the transmitter content during pregnancy (PAPERS 6 and 7) a series of experiments was started in which rabbits were subjected to treatment with various hormones In the first part of this series rabbits were given 17 β estradiol The various reproductive organs were then analyzed histochemically and chemically for changes in the neuronal transmitter content the heart being used as control tissue

The total content of noradrenaline was found to be markedly increased in

the uterus and vagina in animals treated daily with 17 β estradiol (0 5 μg/kg) for 14 days the transmitter content remained at a high level during an experimental period of 14 days even though treatment with estrogen was interrupted already after 7 days It was more difficult to show a clear cut increase in the noradrenaline content of the oviduct This was probably not due to too small a dose of estrogen because even with a much larger dose (50 μg per animal daily for 8 days) BRUNDIN (1965) was unable to demonstrate any certain increase 17 β Estradiol treatment did not influence the noradrenaline content of the ovary or of the heart The increased noradrenaline content of the uterus and vagina manifested itself as an increased number of fluorescent nerves In spite of the enlargement of the organs following the hormone treatment, the density of the adrenergic nerve plexus of the smooth muscle coats remained unchanged or was even more pronounced than in untreated animals Furthermore the fluorescence of the individual axons especially of their varicosities was distinctly more intense than in untreated animals This gave the fluorescent nerve terminals a swollen and clumsy appearance

Judging from these fluorescence microscopic findings it would seem reasonable to assume that the estrogen treatment resulted in an increased transmitter content of a large number of otherwise non fluorescent axons as well as of nerves which are *fluorescent even in untreated animals The actual cause of* this increase is still obscure

The changes in the neuronal noradrenaline level during pregnancy (PAPERS 6 and 7) and estrogen treatment (PAPER 8) do not seem to involve all sympathetically innervated organs but only certain parts of the female reproductive tract It should be recalled that parts of the female genital tract receive their sympathetic innervation by way of functionally unique so-called short adrenergic neurons (PAPER 5) It is possible that some of the changes found in these parts of the genital tract during certain stages of pregnancy and after estrogen treatment represent a further functional parameter distinguishing short adrenergic neurons from ordinary long adrenergic neurons arising in pre and paravertebral ganglia

GENERAL SUMMARY

The distribution and content of certain monoamines—5 hydroxytryptamine dopamine noradrenaline and adrenaline—in the female reproductive tract of guinea pig rabbit, cat and man were investigated with a combination of fluorescence histochemical and spectrofluorimetric methods

Of the monoamines studied only noradrenaline and 5 hydroxytryptamine were present in demonstrable amounts

5 Hydroxytryptamine was found in a system of flask shaped cells in the vaginal vestibular epithelium of rabbit and cat

A very intense catecholamine fluorescence occurred in scattered small branching cells of the chromaffin type

Noradrenaline was found to be concentrated in plexuses of adrenergic nerve terminals with a characteristic variation in density from organ to organ in the various species The terminal network was on one hand related to blood vessels On the other hand the oviduct uterus and vagina had a rich supply of adrenergic nerves in the smooth muscle coats and the ovary contained non vascular nerves in the parenchyma

In denervation experiments the adrenergic innervation was shown to originate from three sources the lumbo sacral ganglia of the sympathetic chain the inferior mesenteric ganglia via the hypogastric nerves and peripheral adrenergic ganglion formations in or near the utero vaginal junction The latter ganglion formation has the same peripheral localization as the cell bodies of postganglionic parasympathetic neurons and thus give rise to short adrenergic neurons contrasting with ordinary long adrenergic neurons from pre and paravertebral ganglia The contribution of each of these three sources to the different parts of the reproductive tract has been elucidated

In a model experiment in which the disappearance and reappearance of the adrenergic transmitter after a single dose of reserpine was followed in organs innervated by short or long adrenergic neurons it was demonstrated that the two neuron types differed functionally in that the transmitter disappeared slower and returned earlier in the short than in the long adrenergic neurons

During the first part of pregnancy there was a prominent increase in the noradrenaline content in the oviduct uterus and vagina of the rabbit A marked increase was registred in the uterus and vagina also after treatment of non pregnant rabbits with 17 β estradiol whether the animals were intact or

oophorectomized The increased noradrenaline content manifested itself as an increased number of adrenergic nerves with a clearly visible fluorescence which may have been due to an increased transmitter content of the nerves in these organs The noradrenaline level of the heart and ovary remained unchanged

Towards the end of pregnancy a marked decrease in uterine noradrenaline was recorded a finding in agreement with the persistence of only few adre nergic nerve terminals The decrease was less evident in the vagina, and did not occur in the oviduct

The present investigation has shown that the female reproductive tract is supplied by two types of adrenergic neurons differing from one another in morphological and functional characteristics A pronounced variation in the transmitter content can be registered in certain parts of the genital tract during pregnancy and after estrogen treatment

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 306

PULMONARY DEFENCE MECHANISMS TO AIRBORNE BACTERIA

BY

RAGNAR RYLANDER

STOCKHOLM 1968

her *Inhabitants* breaths nothing but an impure and thick *Mist* accompanied with a fuliginous and filthy vapour which renders them obnoxious to a thousand inconveniences corrupting the *Lungs* and disordering the entire habit of their Bodies so that *Catharrs Phthisicks Coughs* and *Consumptions* rage more in this one City than in the whole Earth besides

John Evelyn

Fumifugium 1661

‘ Bacterium—kinds of single celled microscopic organism found almost every where

The Pocket Oxford Dictionary of Current English 1957

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INTRODUCTION

The respiratory tract is continuously exposed to airborne microorganisms of a size allowing penetration to the lower parts of the lung Also under certain conditions fluid containing microorganisms can be transferred from the mouth down to the lung In spite of this continuous environmental challenge bacteria normally cannot be cultured from the mucus of the bronchial tree

Exposure to certain agents e g toxic gases and air pollution especially when rich in dust may lead to conditions such as chronic bronchitis where bacteria in large numbers can be found in the air passages The bacterial species found are often of the same kind that usually can be found in the mouth and which under normal conditions are non pathogenic

Since changes in the pulmonary defence mechanisms against bacteria have widespread clinical implications such as the situations referred to above methods for the *in vivo* measurement of the efficiency of the defence mechanisms are of importance

The aim of this work has been to study the relative role of the two main mechanisms responsible for the pulmonary defence against inhaled bacteria namely the mechanical elimination brought about by the mucous transport and the bactericidal activity The study was conducted with non pathogenic bacteria using normal animals and animals which had been exposed to conditions capable of altering the above defence mechanisms

After a general consideration of the elimination of particles and bacteria from the lung a literature review of earlier work dealing with the pulmonary defence against inhaled bacteria is presented In a subsequent chapter, the general principles for measuring separately the mechanical elimination and the bactericidal activity are presented An experimental model for the study of the two mechanisms is suggested and the experimental requirements are discussed The methods are described and examined for accuracy and sensitivity and in the following chapter the results from normal animals are presented Then alterations of the defence mechanisms are given a general consideration and results from experiments where animals have been exposed to various agents are discussed Finally a general discussion concerning the methods and the results is presented

A detailed description of equipment and techniques used is found in the appendix Reference to this description is indicated by * The individual numerical values upon which the figures are based are found in the figure table section

GENERAL CONSIDERATIONS

The ability of the lung to clear itself from deposited matter of various kinds has been extensively studied during recent years and it would be beyond the scope of this work to review in detail all information available concerning the elimination of particles especially as thorough reviews on the subject have been published previously

Recent publications include the work by Hatch and Gross (1964) and by the Task Group on Lung Dynamics for the International Radiation Protection Commission (Bates et al 1966) The reader is referred to these works for a more detailed discussion of the general aspects of the problem

It has been shown that the fate of the individual particle is dependent upon the site of deposition which in turn is determined mainly by the particle s aerodynamic size and to a certain degree by air flow patterns in the airways Particles which deposit above the respiratory bronchioli are eliminated from the lung mainly by means of the mucous transport—this elimination can be referred to as the initial mucous transport The cough mechanism also has been shown to assist in the elimination of particles on the mucus

The mucous transport is determined by the ciliary beat efficiency and the physical properties of the mucus Particles on the mucus are carried upwards into the larger airways and are finally eliminated from the lung via the trachea The amount of material removed is largest immediately after the deposition of the particles and declines rather rapidly after the first hours although small amounts of particles will be eliminated via the mucus for several weeks after exposure

Particles which deposit in or below the region of the respiratory bronchioli become subject to phagocytosis where the uptake by the phagocyte is greatly influenced by the physico chemical properties of the particle Such particles can be transported up into the mucus with or without prior phagocytosis This transport could be due to a movement of fluid from the alveoli up to the mucus—surface active agents seem to play a major role in this connection Secondary penetration from the deposition site in the mucus down into alveoli may take place also

Elimination of particles within phagocytes can be brought about by the mucous transport by disintegration within the cells or by penetration into the blood or lymph vessels Some results suggest that particles inside phagocytes which are situated in the lung tissue might be expelled later into the mucus further up in the respiratory tree

The elimination of particles deposited in the respiratory bronchioli or in the alveoli via either the mucus or the internal routes involves long periods of time and total elimination might not be achieved until several years after the deposition

In the case of bacteria the elimination mechanisms are obviously the same as for other particles and a change in the functioning of any of the mechanisms involved would just as is the case with other particles expose the tissue to the bacteria for a longer or shorter time As the occurrence of a biological effect caused by bacteria often requires multiplication, mechanisms for preventing this event are important Such mechanisms are

1 The mechanical removal of bacteria from the lung The number of bacteria transported out of the lung per unit time in the immediate post exposure period is of interest as well as the state of bacteria when transported i e whether they are inside or outside phagocytes

2 The killing of the bacteria or the prevention of multiplication of bacteria remaining in the lung

EARLIER STUDIES ON THE ELIMINATION OF BACTERIA FROM THE LUNG

The following general review of the literature dealing with the pulmonary defence against inhaled bacteria is supplied as a background for the experimental work on the separate measurement of the mechanical and bactericidal defence mechanisms

Several different approaches have been made to study the elimination of bacteria from the lung In most of the early works the presence of bacterial cells has been studied using histological sections stained for bacteria Although in a few cases viable counting also has been performed most of these studies estimate the elimination only in a qualitative way In later experiments quantitative information has been obtained with the aid of techniques which determine the number of viable bacteria present in the lung at various times after the exposure Studies on factors influencing the pathological effects of bacteria deposited in the airways can be looked upon as indirect studies of the defence mechanisms

This arbitrary classification of the extensive literature dealing with the elimination of bacteria will be used in the following to increase the clarity of this review

1 STUDIES ON BACTERIA DEPOSITED IN THE AIRWAYS

1 1 Elimination of bacterial cells

The early works in this field have been summarized by Tchistovitch (1889) who also reported his own studies on different bacteria species in stained histological sections of lungs from rabbits which received bacteria intratracheally Referring to the works by Muskatblüth (1887), Lahr (1887) and Buchner (1888) he found that contradictory opinions existed as to the fate of intratracheally injected bacteria The findings from his own studies were that different species of bacteria were treated differently by the lung—the most pathogenic organisms were not found inside macrophages or leucocytes but could be cultured from the blood whereas less pathogenic organisms could mainly be found intracellularly and not in the blood His findings partly explained the discrepancies between the results of earlier authors and

have been confirmed in several experiments performed later (e g Terrell et al 1933, Rake 1936 Cannon and Walsh 1937)

Briscoe (1907) injected staphylococci and pneumococci intratracheally in guinea pigs and studied the bacteria in histological sections Cultures were taken from heart blood and lungs He concluded that the majority of the non pathogenic bacteria were taken into phagocytic cells during the first ninety minutes Pneumococci were phagocytized to a lesser degree although some what better in immunized animals

Kettle (1930) studied lung sections from animals which had received tubercle bacteria intratracheally He compared animals who received only bacteria with others who received silica dust in the same injection and found a larger number of bacteria in lung sections from the latter group In addition more of those animals died of tuberculosis

Harper and Morton (1962) studied the elimination of inhaled radioactive bacterial spores in guinea pigs killed various times after exposure The lungs were digested in nitric acid and the amount of radioactivity was measured Control experiments showed that the radioactivity was firmly bound to the spores and was probably not removed during the observation period Tak ing the radioactivity as a measurement of the number of spores they found that the elimination rate was very slow—after 11 days 59 % of the initial dose remained Since their measurements were not initiated until one day after exposure the early effect of the mucous transport was not measured but only the long term elimination via the mucus or via internal routes

Green and Kass (1964 a) studied the elimination of *Staphylococcus aureus* and *Proteus mirabilis* from the lungs of mice The animals were exposed to an aerosol and histological sections of the lungs were taken at various time intervals after exposure The sections were stained using ordinary and immuno fluorescent techniques Some experiments were performed with bacteria marked with ^{3} P and the amount of radioactivity present in the lung was determined In addition bacteria in homogenates of the lung were quantita tively cultured

The authors found that the radioactivity of the lung declined 14—20 % during the first 4 hours after exposure, whereas the number of viable bacteria declined 80—90 % and concluded that bacteria remain in the lung some time after they have lost their viability They also concluded that physical removal of free bacteria accounted for a relatively small fraction of the total loss of viable bacteria under the experimental conditions present They further suggested that the action of the mucociliary stream might be largely related to the transport from the lung of phagocytes containing material of bacterial origin These conclusions were based upon findings using the immuno fluorescent technique on sections where only few bacteria were judged to be

outside phagocytes in the alveoli and on the respiratory epithelium whereas many were found inside phagocytes

1 2 Reduction of viable bacteria

One of the earliest works in this field was reported by Stillman After initial experiments (1923) in which the presence of bacteria in lungs of mice following inhalation of an aerosol was ascertained exposures to aerosols of pneumococci haemolytic streptococci and *Bacillus influenzae* were carried out (1924)

In normal animals no pneumococci could be cultured from small pieces of lung, nor from blood samples 4 hours after exposure In animals treated with alcohol viable bacteria were found in the lung as long as 5 days after the exposure An increased mortality also was noted in the alcohol treated group Similar but less striking results were obtained when streptococci and *B influenzae* were used

Cannon and Walsh (1937) studied the fate of non virulent staphylococci and virulent pneumococci which were instilled intranasally in rabbits and guinea pigs The presence of bacteria was determined by culturing the lung liver and spleen and blood samples They concluded that bacteria administered in a suspension could penetrate from the nose into lungs from where they sometimes entered the blood Bacteria also could be cultured from the liver and the spleen In intranasally vaccinated rabbits the aspiration still took place but the dissemination from the lungs to the blood was restricted and the growth of the bacteria in the lung and the body as a whole was inhibited

Cralley (1942) exposed rabbits to an aerosol of *Serratia marscesens* and studied the number of viable bacteria in lung and trachea crushings separately He found that only 20 % of the initial number of bacteria could be cultured from the lung 1 hour after exposure and concluded that 80 % were removed Animals were exposed to changes in temperature and humidity and the author found that the extreme and sudden changes studied exerted a depressing effect on the removal No attempt was made to explain the defence mechanisms in operation

A study on the fate of bacteria applied on tracheal epithelium was reported by Dold (1943) who used cultures of *Bacillus prodigiosus* and *Bacillus anthracis* on *in vitro* preparations of rabbit trachea No outgrowth of bacteria occurred by 24 hours after the application A heavy outgrowth occurred on heat-treated tracheas In this connection it is interesting to note that saliva has been shown to possess important bactericidal activities — several of these studies were reviewed by Rolla (1964)

Barnes (1947) determined the number of anthrax spores present in homogenates of lung stomach and lymph glands of rabbits guinea pigs and mice at different times after exposure Using the viable count technique he found that

used to study the influence of hypoxia, cold stress and ethanol intoxication When various species of bacteria were compared the reduction of viable bacteria was found to differ *Staphylococcus albus* showed the most rapid rate, *S aureus* an intermediate and *P mirabilis* the slowest rate of reduction The authors concluded that the observed difference might represent the varying susceptibility of the bacteria to phagocytosis and intracellular digestion

The effect of the treatments used also varied according to the bacterial species examined The reduction of viable *S albus* and *S aureus* which normally was rapid in the lung showed a significant depression after ethanol treatment For *P mirabilis* which normally had a low reduction this was completely inhibited By contrast hypoxia affected very little the reduction of viable proteus but depressed significantly that of staphylococci Cold stress induced by placing the animals in a chamber at 13—17 with their fur kept wet had an approximately equal inhibitory effect on the three species

The authors suggest that the interaction of the relative resistance of individual bacteria the relative susceptibility of the pulmonary macrophage to environmental changes and the operation of an oxygen dependent system in macrophages are factors responsible for the observed results The possible specific effects of the various treatments on the mucous transport mechanism were not discussed

Laurenzi and Guarneri (1966) who also noted the effect of ethanol on the reduction of viable bacteria in a separate experiment measured the mucous transport rate in trachea of kittens and found that ethanol decreased the transport rate They also found that ethanol interfered with the mobilization of alveolar macrophages in mice exposed to bacterial aerosols but were unable to show any effect on phagocytosis using an *in vitro* system

Laurenzi et al (1965) reported on the effect of tobacco smoke and intravenously injected carbon particles—the former treatment had a depressing effect and the latter treatment had no measurable effect on the reduction The presence of ordinary gram negative pathogens in the intestinal flora of the mouse was shown to predispose to smoke interference with the pulmonary disposal of inhaled bacteria

The effect of acute renal failure on the reduction of viable *S aureus* was reported by Goldstein and Green (1966) whose results indicate that metabolic disturbances is another factor which might influence the reduction of viable bacteria in the lung

Kass et al (1966) reported an inhibition of clearance of staphylococci from mice lungs after virus inoculation The effect usually occurred towards the end of the first week and lasted 1 to 3 days

The pulmonary clearance of various gram negative bacteria from the lungs of mice was studied by Jackson et al (1967) They reported a difference in

clearance rates between two strains of pseudomonas as compared to e g *E coli* and *P mirabilis* A significant increase in lung weight due to broncho pneumonia was found in the animals exposed to pseudomonas

2 STUDIES ON EFFECTS OF BACTERIA DEPOSITED IN THE AIRWAYS

Numerous animal experiments have been performed to test the conclusions drawn from epidemiological studies where a correlation between exposure to dust or gases and the incidence of respiratory disease has been found In several of these experiments bacteria have been used and the effects due to the bacteria have been compared in normal animals and animals exposed to e g toxic gases or air pollution Therefore these experiments can be looked upon as indirect measurements of the pulmonary defence mechanisms to bacteria

The works in this area contain studies using several different organisms and a division of the works into the following groups is convenient

1 Tuberculosis and related infections
2 Pneumonia
3 Other infections

2 1 Tuberculosis and related infections

Cesa Bianchi (1913) exposed guinea pigs to various types of dust for 2—4 hour intervals for 8—10 weeks Together with appropriate controls the animals were then exposed to tubercle bacteria by means of subcutaneous intraperitoneal intravenous or intratracheal injections Conclusions drawn from these experiments were that dust exposed animals had a lowered resistance to tubercle bacteria and that they all developed tuberculosis in the lungs irrespective of how the bacteria were administered All types of dust tested gave about the same effect including inert dusts such as talc and cement

This and several other works where essentially the same results have been found were reviewed by Tacquet et al (1966) These authors also reported on their own experiments where the effects due to intratracheal injection of *Mycobacterium kansasii* in guinea pigs were studied Pulmonary X rays and macro- and microscopical examinations of lung tissue showed that the administration of coal and silica dust enhanced the pathological effect of the bacteria These findings are in agreement with earlier works by Policard and Dufourt (1937 Gernez Rieux et al (1959) and Tacquet et al (1963) where essentially the same techniques have been used

Durck (1896) reported studies where rabbits were challenged with an aerosol of bacteria isolated from clinical cases of pneumonia If bacteria only were administered no disease occurred but if dust was given intratracheally simultaneously all animals developed pneumonia

Sisson and Walker (1915) used cats and produced lobar pneumonia by intrabronchial insufflation of pneumococci If the lungs of the animals had been exposed to ether vapours intravenous injections of the bacteria were found to produce pneumonia

Rake (1936) inoculated mice intranasally and found that the pneumococci reached the lower respiratory tract and the alveoli almost immediately and that invasion of the blood occurred rapidly He also found that the incidence of disease was dependent upon the strain and type of organism and the breed of mice used

The influence of mucin on pneumonia was studied by Nungester and Jourdonais (1936) They used rats which received intratracheal injections of pneumococci and noted an increase in the number of animals developing pneumonia if 0 1 ml sterile mucin was added to the inoculate

In similar experiments (Nungester and Klepser 1938) no pneumonia cases occurred in normal animals whereas 80 % of the animals who 24 hours prior to the bacteria challenge had received an intratracheal injection of mucin developed pneumonia The effects of alcohol, cold stress and anesthesia were also tested—an increased incidence of pneumonia was found in the treated animals

The susceptibility to lobar pneumonia after intrabronchial injections of pneumococci has been studied in rats exposed to various types of dust Baetjer and Vintinner (1944) found that previous exposure to silica and feldspar dust increased the resistance to pneumonia as measured by incidence of infection mortality and case fatality rates In later experiments Portland cement dust (Baetjer 1947) bituminous coal dust and combustion smoke thereof (Vintinner and Baetjer 1951) and aluminium dust (Vintinner 1951) were found not to increase the incidence of pneumonia

The effect of a previous viral infection on the outcome of subsequent challenges with pneumococci has been studied in several reports Harford et al (1949) and Gerone et al (1957) both presented reviews of earlier research on this particular problem together with results from their own experiments A general finding in all these studies is that viral infections decreased the normal pulmonary defence against bacteria The presence of extensive viral necrosis in the bronchial epithelium was described by Harford et al and was considered to be of importance for the lowered resistance

An experimental model where animals were exposed to an aerosol of pathogenic bacteria and the mortality in a control and a treated group was compared was first reported by Miller and Ehrlich (1958 a) They found (1958 b) that exposure to ozone (1—4 ppm) significantly reduced the resistance of mice to subsequent respiratory infection due to *Klebsiella pneumoniae* Similar results were obtained with hamsters

Purvis et al (1961) studied the effect of exposure to low concentrations of ozone before or after the exposure to *K pneumoniae* If 4 ppm ozone was given 1, 3 and 6 hours before the bacteria an increased mortality was found whereas ozone exposure 19 and 27 hours before had no demonstrable effect The extent of bronchial pneumonia however, was not found to be significantly higher in mice exposed to both ozone and bacteria than in mice exposed to the infectious aerosol only The authors conclude that "it may be the irritant temporarily affects the ciliary movement of the mucus flow in the respiratory tract"

Using the same experimental model Ehrlich and Mieszkuc (1962) reported on the effect of simulated high altitude Their results indicated that a 3 days exposure to a simulated 18 000 feet altitude increased the resistance to the infection After 7 days this increase disappeared and the resistance was significantly reduced after a 30 and 90 days exposure

Ehrlich (1963) continued the studies on ozone and found that exposure of mice to 4 ppm ozone 3 6 and 27 hours after bacterial exposure significantly enhanced the mortality and shortened the survival time The effect of nitrogen dioxide also was reported in this paper and at exposure levels of 25 ppm for 2 hours essentially the same results as for ozone exposure were obtained

The results from long term exposure to low concentrations of NO were reported by Ehrlich (1966)—a 6 month s exposure to 0 5 ppm was found to decrease the resistance of mice to a challenge with the klebsiella aerosol

2 3 Other infections

Abbot (1896) studied the death rate in rabbits exposed to *Streptococcus pyogenes*, *S aureus* or *Escherichia coli* He found that acute alcohol intoxication markedly increased the death rates for animals exposed to *Strep pyogenes* and to a lesser degree for animals exposed to *E coli* In alcohol treated animals which were challenged with *S aureus* no marked difference was found

Janssen et al (1963) reported on the synergistic activity between influenza virus and *S aureus* in guinea pigs The animals were exposed to an aerosol of virus or virus and bacteria An increased mortality was observed in the animals which received both virus and bacteria Synergism was observed only in

animals exposed to staphylococci within 24 hours after exposure to influenza virus but did not occur if the virus followed the bacteria

Miller and Ehrlich (1958 a) compared the mortality in groups of hamsters and mice challenged with streptococci where one group had been exposed to ozone An increased mortality rate was found in the ozone exposed animals

Coffin and Blommer (1965) studied the influence of cold following ozone exposure on the mortality of mice exposed to streptococci In later experiments (Coffin 1966) the effect of NO exposure was reported using the same experi mental model Both treatments were found to increase the death rate

Exposure to irradiated automobile exhaust was also shown to increase the mortality after challenge to streptoccoci (Coffin and Blommer 1967)

3 COMMENTS

It is necessary to point out that a certain confusion exists concerning the terminology in this field Several authors have studied the reduction of viable bacteria in the lung and referred to that as "clearance , ' elimination or "disposal of bacteria others have used the same terms when studying the disappearance of bacterial cells which could be viable or non viable when observed In experimental models where the disease producing effect of certain bacteria has been compared in normal and treated animals the terms elimination or clearance have been used indistinctly in the conclusions concerning a possible influence on the defence mechanism

In order to clarify the situation the following terminology has been used throughout this work

Elimination The bacterial cells are no longer present in the lung due either to mechanical transportation out of the lung effected by the mu cous transport and by phagocytes which can migrate to other parts of the body or to disintegration within the lung brought about by the lytic activ ity of phagocytes or extra cellular enzymes

Reduction of viable bacteria The bacteria cannot be cultured due to the in fluence of the bactericidal and bacteriostatic mechanisms of the lung However they still may be present in the lung as structurally intact cells Measurements of viability can be made with or without prior disintegration of phagocytes

Conclusions drawn from studies on the defence of the lung against bacteria as reviewed above agree that non pathogenic bacteria which deposit in the

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Conclusions drawn from studies on the defence of the lung against bacteria as reviewed above agree that non pathogenic bacteria which deposit in the

airways are rapidly eliminated or rendered non viable under normal circumstances Pathogenic organisms have been found to remain longer in the lung and it has been shown that they can penetrate beyond the lung reaching other organs of the body fairly rapidly in spite of the defence of the lung Non pathogens on the other hand do not usually penetrate beyond the lung and the pulmonary lymphnodes

The relative importance of the mechanical elimination of bacteria is dependent upon the deposition site the more bacteria that deposit on the mucus the more are eliminated mechanically However, the intra or extra cellular state of the bacteria when eliminated via this mechanism has not been clearly characterized and contradictory conclusions can be found Green and Kass (1964 a) reported that only a few bacteria studied in histological sections of lungs were found to be outside phagocytic cells within a relatively short time after deposition Against this stands the findings by Cralley (1942) who using a preparation technique which apparently did not destroy the phagocytes was able to cultivate bacteria from tracheal epithelium at varying times after the exposure

Many of the studies reviewed have shown that environmental factors might alter the defence of the lung to bacteria This influence has been demonstrated as an alteration in the reduction of viable bacteria of various bacterial species or as a change in the severity or incidence rate of a pathological effect caused by the bacteria In this area also contradictory conclusions concerning the effect on the different defence mechanisms can be found For instance, ethanol has been found to affect the reaction of the lung towards bacteria in experiments by several authors Green and Kass (1964 b 1965) discussed extensively the inhibitory activity on the reduction of viable bacteria found in ethanol treated animals and inclined to attribute the change to an effect on the phagocytes

In other studies it has been shown that ethanol can decrease the mucous flow rate in the trachea (Lomell 1908), an effect which would induce an alteration in the reduction of viable bacteria if preparations of the whole lung were studied Laurenzi and Guarneri (1966) found a marked decrease of the mucous transport function in the trachea of kittens rendered unconscious by intraperitoneal injections of ethanol They reported further that although ethanol interfered with the mobilization of alveolar macrophages no effect of ethanol on macrophage metabolism or phagocytosis could be detected when these processes were studied *in vitro* Their results are in agreement with results from experiments by Parkinson and Cantab (1909) who studied bacteria in the peritoneal cavity and found that ethanol did not affect the phagocytic process until it was present in such high concentration that the viability of the cell was endangered

Contradictory conclusions also can be found concerning the effect of dust on the pulmonary defence against bacteria In a series of inhalation experiments Baetjer and Vintinner (e g 1944 1951) found that dust did not increase the susceptibility of animals to lobar pneumonia On the other hand Cesa Bianchi (1913) and Tacquet et al (1966) among others found that the lesions due to an infection with tubercle bacteria were more severe in animals which had been exposed to dust

4 CONCLUSION

Although much information is available concerning the defence of the lung as a whole towards bacteria the relative importance of the different mechanisms responsible for the killing or elimination of bacteria has been studied only little under *in vivo* conditions This deficiency is true especially in experiments where the influence of various conditions has been evaluated Several of the conditions used also have the potential ability to influence separately the mechanical elimination by the mucous transport and the bactericidal mechanism

Separate measurements of the two defence mechanisms and how they are influenced by various conditions are therefore of importance and could provide information helpful for the understanding of the mechanisms behind the occurrence of acute and chronic respiratory disease It appears to be of special interest to conduct studies during the first hours after exposure when the eventual onset of multiplication of the inhaled bacteria is determined

METHODS

1 GENERAL CONSIDERATIONS

At any given time (t_1) after deposition in the lung at time zero (t_0) viable bacteria can be found in the following fractions

Viable bacteria (V)

V_L — viable bacteria in lung
V_T — viable bacteria eliminated via the trachea
V_I — viable bacteria eliminated via interstitial routes

Dead bacteria (D)

D_L — dead or disintegrated bacteria in lung
D_T — dead bacteria eliminated via the trachea
D_I — dead bacteria eliminated via interstitial routes

Bacteria in the above fractions can be found extracellularly or inside phago cytes

Assuming no multiplication of the inhaled bacteria the reduction in the number of viable organisms at the time t_1 can be expressed as

$$V_L(t_0) - V_L(t_1) = V_T + V_I + D_T + D_L + D_I \qquad (A\ 1)$$

For non pathogenic bacteria V_I can be assumed to be zero in normal animals The viability loss for non pathogens during the first few hours after exposure is thus

$$V_L(t_0) - V_L(t_1) = V_T + D_T + D_L + D_I \qquad (A\ 2)$$

The reduction in the number of viable bacteria from the lung is brought about by mucous transport and the bactericidal mechanisms of the lung The mechanical component is represented by V_T' in equation (A 2)

For experimental purposes the importance of the mucous transport in the reduction of viable bacteria from the lung (m) can be estimated as the per cent of viable bacteria eliminated from the lung via the trachea (V_T)

$$m\ (t_1) = \frac{V_T \quad 100}{V_L(t_0) - V_L(t_1)} \qquad (A\ 3)$$

Low values of 'm' implies that the mechanical elimination of viable bacteria during a given period was negligible

Values of "m" close to 100 implies that this was the only mechanism responsible for the reduction of viable bacteria during that time period

In cases where multiplication of the inhaled bacteria takes place within the time of the experiment, 'm' might reach even higher values

If insoluble particles are inhaled in the lung together with the viable bacteria the particles will be found in the following fractions at any given time (t_1) after deposition (t_0)

Insoluble particles (P)

P_L — Particles in lung
P_T — Particles eliminated via the trachea
P_I — Particles eliminated via interstitial routes

The particle elimination at any given time (t_1) can be expressed as

$$P_L(t_0) - P_L(t_1) = P_I + P_T \qquad \text{(A 4)}$$

For particles of a kind within which elimination via interstitial routes is negligible the amount eliminated via the trachea will represent the total loss from the lung

$$P_L(t_0) - P_L(t_1) = P_T \qquad \text{(A 5)}$$

In cases where the insoluble particles have the same characteristics of deposition and mucous elimination as the viable bacteria the number of insoluble particles eliminated via the trachea (P_T) will always be proportional to the total number of bacteria eliminated via this route ($D_T + V_T$)

If no reduction in the number of particles in the lung has occurred at any given time after deposition the fraction P_T is zero (equation A 5) The values of D_T and V_T (equation A 2) will be zero also and a reduction of viable bacteria will be entirely due to the bactericidal mechanisms of the lung Such a case is illustrated in figure 1 a (next page)

If a particle reduction takes place P_T will have a value depending on the degree of the reduction while $V_T + D_T$ will attain a proportional value A difference between the particle reduction and the reduction of viable bacteria will be due to the bactericidal mechanisms of the lung This difference, however is not necessarily a measurement of all the bactericidal activity since some or all of the bacteria which are eliminated via the trachea may have been killed by the bactericidal mechanisms prior to elimination via the mucus

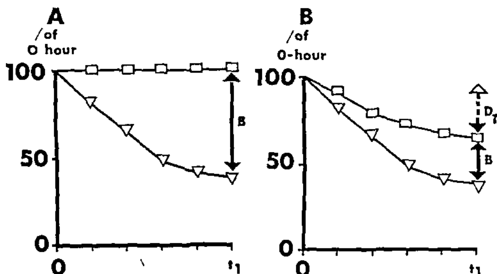


Figure 1 Insoluble particles (□) and viable bacteria (▽) with equal deposit and mucous transport characteristics found in lung at a given time (t_1) after deposition Representative results

at the time of the measurement (D_T in equation A 2 and in figure 1 b) Thus the relative importance of the mechanical elimination of non pathogenic, viable bacteria versus the bactericidal mechanisms can be estimated only by using equation A 3

2 EXPERIMENTAL PROCEDURES

The relative importance of the mechanical elimination of viable bacteria (equation A 3) was studied in guinea pigs exposed to an aerosol of insoluble particles and non pathogenic viable bacteria The number of particles and bacteria within the lung was determined various times after exposure

Determinations of the number of particles and bacteria present were made in whole lung homogenates and in the trachea by flushing the inside or homo genizing tracheal sections of a standardized length

Killed non pathogenic bacteria labelled with ^{35}S were used as insoluble particles and the number present in the various preparations was estimated with an autoradiographic technique Non pathogenic bacteria were used for the viable assay and the number was determined using the pour plate technique for viable count

To make the deposition and elimination conditions as equal as possible the killed bacteria used as insoluble particles were of the same species as the viable bacteria

2 1 Measurement of reduction of viable bacteria $V_L(t_0) - V_L(t_1)$

For the measurement of the reduction of viable bacteria the number of viable bacteria in the whole lung was determined immediately after exposure and at various time intervals thereafter

2 2 Measurement of viable bacteria eliminated via trachea V_T

The number of viable bacteria eliminated via the trachea (V_T) can be expressed as

$$V_T = f \quad \overline{v}_T \qquad (B\ 1)$$

where f is a factor expressing flow efficiency and $\overline{v}_T$ is the mean number of viable bacteria present in a standardized section of trachea within the time interval $\Delta t = t_1 - t$

To determine f , the following equation was used based upon the assumption that the insoluble particles were eliminated from the lung via the trachea (equation A 5)

$$P_L(t_1) - P_L(t\ ) = f \quad \overline{p}_T$$

$$\text{or} \qquad (B\ 2)$$

$$f = \frac{P_L(t_1) - P_L(t\ )}{\overline{p}_T}$$

where $\overline{p}_T$ is the mean number of particles present in a standardized section of trachea within the time interval $\Delta t = t_1 - t_2$

The number of insoluble particles present in the whole lung was measured at different times after exposure to the aerosol ($P_L(t_1)$ (t) etc) Measurements of the number of particles present in trachea sections were performed in the same animal at the same time intervals (t_1 t etc) and a mean value was calculated for various periods after exposure ($\overline{p}$ ($t_1 - t$) (t $- t_3$) etc) With the aid of the values obtained in these measurements f was determined using equation (B 2)

Since the insoluble particles and the viable bacteria have the same mucous elimination characteristics the flow efficiency value (f) for the particles from equation (B 2) can be used for the viable bacteria in equation (B 1)

To determine $\overline{v}_T$ the number of viable bacteria present on the inside of tracheal sections was determined various times after exposure to the bacterial

aerosol A mean value ($\overline{v}_T$) was calculated for various periods after exposure (t_1-t, t_2-t_3 etc)

3 VALIDITY OF MEASUREMENTS

The following requirements must be met if measurements of the mechanical elimination of viable bacteria by the experimental procedure outlined above can be interpreted according to equation (A 3)

1 The killed, radioactive bacteria used as particles must be resistant to lysis in the lung This requirement has been subject to experimental analysis in the subsequent chapter

2 The killed radioactive bacteria and the viable bacteria must have the same deposition characteristics when inhaled into the lungs This requirement also has been subject to experimental analysis in the subsequent chapter

3 No elimination of killed radioactive bacteria or viable bacteria must take place via interstitial routes This requirement has been experimentally controlled

4 The mechanical elimination of bacteria by means of mucous transport must be equal for killed, radioactive and viable bacteria

The elimination of particles deposited on the mucus is entirely a mechanical process, provided that lysis effected by enzymes in the mucus does not take place, and free viable and dead bacteria will be eliminated at equal rates

A difference in transport rate could theoretically be present if the two kinds of bacteria were taken up by phagocytes to a different degree with a resulting difference in particle size Although particles deposited on the mucus of the respiratory epithelium are not considered to be taken up by phagocytes to any large degree (Robertson 1941) one can consider an extreme hypothetical case where either only non viable or only viable bacteria deposited on the mucus are immediately taken up by phagocytes whereas the other type of bacteria remains free The mucous transport would then have to deal with particles in the size range of free bacteria ($1-2\mu$) and phagocytes ($15-25\mu$) According to results by Antweiler (1958) who was not able to detect any difference in mucous transport time of dust particles of varying sizes this size difference will not result in a difference in mucous transport rate

The amount of radioactivity used in these experiments is negligible compared to the levels which have been shown to affect pulmonary clearance of particles in rats (Cember et al 1961)

4 1 Animals

Full grown male guinea pigs weighing between 250 and 400 g were used They were obtained from ordinary animal dealers stock and fed with water and normal food ad libitum Before being used in an experiment each animal was checked for outer symptoms of respiratory or other disease In case of apparent weight loss or other sign of disease the animal was not used

4 2 Bacteria

In order to select a bacterium which could be considered truly non pathogenic cultures were made from swabs taken from guinea pigs mouths A strain of *Escherichia coli* found to be present in all cultures was used being considered a non pathogenic bacterium to which the animal is continuously exposed under normal conditions The bacteria were harvested from growth on nutrient agar plates and suspended in phosphate buffer for aerosolisation

Radioactively labelled bacteria were obtained by culturing *E coli* in a synthetic medium* which contained 5 mC of ^{35}S per 100 ml After 24 hours at 37 C the *E coli* culture was centrifuged and stored in 15 % formaldehyde An aliquot was taken from this suspension prior to the experiments centrifuged and washed in phosphate buffer three times to remove the formaldehyde and non incorporated ^{35}S The resulting suspension of killed, radioactively labelled bacteria was mixed with viable *E coli* in buffer for aerosolisation A densitometer was used to standardize the concentration of radioactive and viable bacteria in the suspensions used in the experiments

4 3 Aerosol exposure

The animals were exposed for 10 minutes to the bacterial aerosol in a stainless steel cloud chamber* (fig 2 and 3) The chamber housed 8 animals each of which was kept within individual wire mesh cages The aerosol was generated by a Collison atomizer* and subsequently diluted with air in order to facilitate evaporation for formation of droplet nuclei

Microscopic examination of aerosol samples collected on Millipore filters* showed that more than 90 % of the particles in the air consisted of single cells As mono disperse conditions were not considered necessary for these experiments a detailed control of the particle size distribution in the aerosol was not performed in each experiment

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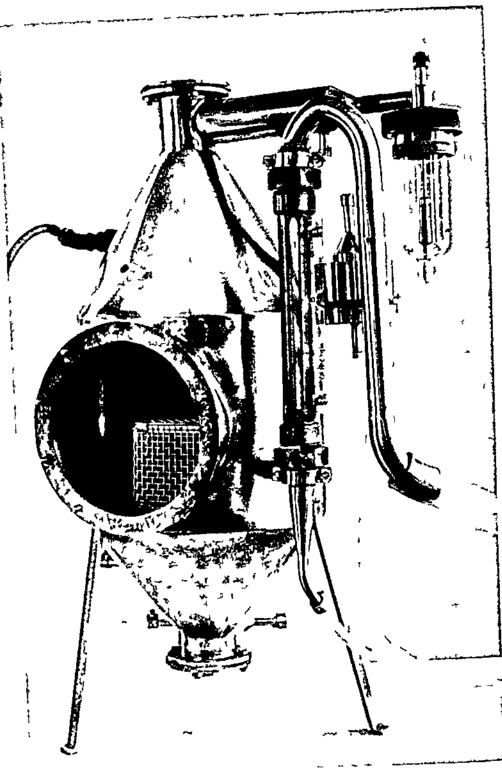
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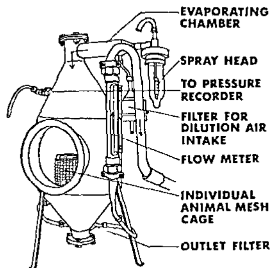


Figure 2 Bacteria exposure chamber

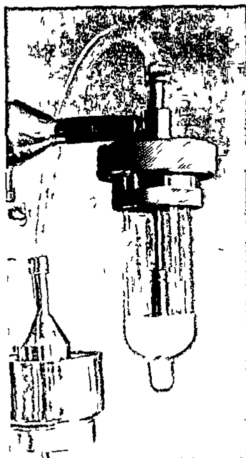
The aerosol flow through the exposure chamber was kept at 3 m³ per hour which was calculated to produce a stable number of bacteria in the chamber air within 0 9 minutes The relative humidity in the chamber during the exposure was found to be 20—45 %

4 4 Preparations

The animals were killed with an intraperitoneal injection of 2 ml sodium mebumal When unconscious they were placed on their backs with the head down to prevent aspiration of particles and bacteria from the mouth Death usually occurred within 1—1 5 minutes after the injection Since the initial mucous elimination is rapid even small variations in the timing of the determinations made at various time intervals after exposure could influence the results A stopwatch was used to minimize the variation in sampling time

4 4 1 *Lung*

The lungs were removed from the thoracic cavity under aseptic conditions and rinsed in sterile phosphate buffer The heart most of the paratracheal lymph nodes and the oesophagus were removed from the preparation and the lungs were ground in motorized glass homogenizers 10 ml of 5 % saponine was added to ensure complete lysis of phagocytes and to prevent conglomeration



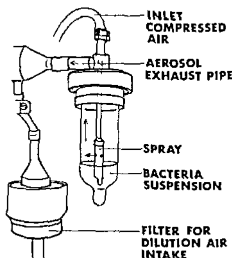


Figure 3 Collision atomizer and aerosol inlet

of particles and bacteria The lungs were ground until completely homogenized with the exception of small fragments of tracheal cartilage

The influence of the homogenized lung and of saponine on the outgrowth of *E coli* was studied in control experiments where the outgrowth of bacteria in nutrient broth was compared with and without the addition of lung homogenate and varying concentrations of saponine No difference was found between the outgrowth of bacteria in the control culture and in saponine concentrations under 10 %

To control that no dissemination of killed, radioactively labelled or viable bacteria took place beyond the lung, blood samples the spleen, liver and kidney were examined at various times after the exposure Radioactive or viable *E coli* could not be detected in these preparations

4 4 2 *Trachea*

Immediately after death, the trachea was uncovered and an 1 cm section was closed at both ends with clamps and removed from the animal The section was either ground in glass homogenizers with 5 % saponine or the inside was flushed with 10 ml of sterile 0 9 % NaCl after the lower clamp had been cut off The lower clamp was chosen to minimize loss of bacteria since the organisms present in the trachea could be expected to be transported on the mucus to the upper end of the trachea, even after sealing had been effected The washings were collected in a sterile glass tube for particle and bacteria determinations When the number of free, viable bacteria in the trachea was to be determined saponine was not added to the washing fluid

The efficiency of the washing procedure in removing free viable bacteria from the trachea sections was tested by determining the number of bacteria found in repeated flushings of the section After the initial washing only a few bacteria could occasionally be found in repeated flushings

To determine the variation of the number of viable bacteria found in tracheal washings at various times after exposure, flushings were made at 10 minutes intervals up to one hour after exposure The mean number from ten experiments was found to decrease slowly and samples taken at half hourly intervals were found to give approximately the same mean value for that time period ($\bar{v}_T$) as samples taken at shorter intervals

4 5 Determination of bacteria

4 5 1 *Viable bacteria*

The number of viable bacteria present in the various preparations was determined using the bacteriological pour plate technique The different preparations were diluted at tenfold increments using 0 9 % NaCl as diluent and a 0 5 ml sample was mixed in endo agar pour plates After incubation at 37 C overnight, the number of *E coli* colonies was counted with the aid of a plate microscope and a touch counter* Duplicate dilution series were made from all preparations one determination was made from each series and the mean value from the two determinations was calculated

Experiments were performed to test the accuracy of the bacterial determination method The error of any single measurement of a preparation was determined by preparing ten plates from the same preparation To insure the proper correlation between concentration of bacteria and dilution this experiment was repeated at four different concentration levels

Table 1

Relative standard deviation of 10 viable count determinations from the same sample 4 different concentration levels of viable E coli

Bacteria level	1	2	3	4
Mean viable count	486	215	36	12
Logarithmic viable count	2 69	2 33	1 55	1 06
Standard deviation	0 03	0 03	0 1	0 08
Relative standard deviation	1 0	1 5	6 2	7 3

The bacterial counts were transformed into logarithmic values before the statistical evaluation shown in table 1

The standard error of a single determination was found to be inversely related to the order of magnitude of bacteria present in the sample Thus provided that the means are calculated as logarithmic values, the sensitivity of the procedure as performed under the present conditions is ± 8 % at a level of around 10 bacteria and ± 7 % at a level of around 500 bacteria

The accuracy of the tenfold diluting of preparations was studied in an experiment where the number of viable bacteria present in the preparation before and after a tenfold dilution was determined Twenty such preparations were studied and the results were subject to statistical analyses The average value of the tenfold dilutions was found to be 9 0 % of the original, with the standard deviation ±2 4 % This difference from the expected 10 % was not statistically significant, however Calculated as the percentage of the mean, the deviation found in this experiment amounts to 27 % which is about four times as large as the error of the single viable count determination

The counting of the colonies on the plates was found to be very accurate—the colonies were clearly visible in the plate microscope and the use of the touch counter diminished the risk for counting errors for the technician Duplicate countings were initially performed but the counting error was found to be negligible and no statistical evaluation was performed on this procedure

Aggregation of bacteria was not considered to cause any error of importance in the viable count determinations as all lung preparations were ground and shaken in 10 ml of 5 % saponine and the tracheal washings were performed with a far larger amount of fluid than the amount of mucus

present Furthermore, the number of bacteria in the lungs was always relatively small which further reduced the risk for aggregation

These results indicate that a main factor contributing to the inaccuracy of the determination of viable bacteria is the variation found in the tenfold dilution series This result agrees with earlier reports by Taylor (1962)

In order to minimize the influence of this error all viable count determinations in the experiments reported here were made on the undiluted preparation fluid or a tenfold dilution Animals were exposed to bacterial concentrations which would yield between 50 and 400 colonies on agar plates at a tenfold dilution which equals 10—80 000 organisms in the lungs

4 5 2 *Killed radioactive bacteria*

The number of radioactive bacteria was determined using an autoradiographic technique similar to one described earlier by Berlin and Rylander (1963)

An 1 ml sample of the preparation fluid was filtered through a Millipore filter* If the lung homogenate was too concentrated the filter would quickly clog up therefore the homogenate was diluted 100 fold before filtration The silicone treated funnel of the filter holder then was rinsed with water three times to wash down particles that might have adhered to the walls The filters were dried at room temperature applied onto X ray film and exposed for around 3 weeks

Before being developed the films were removed from the filters under water in order to prevent electrostatic discharges which could cause blackening of the silver grains in films The films were developed in the usual manner and studied at 30 × magnification with the aid of a plate microscope* The image of the bacteria appeared as easily distinguishable black dots which then were counted (fig 4)

The technical procedures involved in the preparation of the samples i e the tenfold dilution and the sampling are identical to the procedures whose accuracy has been tested using viable bacteria and no further evaluation of the methodological errors connected with these procedures was performed

Experiments were performed to determine the reproducibility and the precision of the counting procedure by letting two observers count the same 10 autoradiograms independently and by letting the same observers count the 10 autoradiograms at two different times The results are found in figure 5 where it can be seen that the difference between two independent

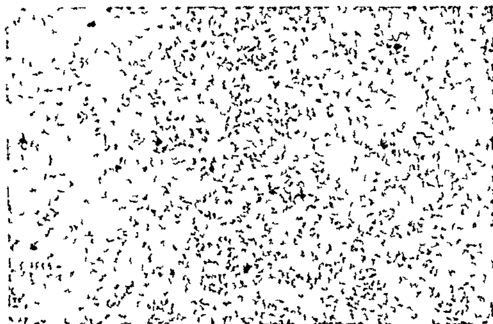


Figure 4 Autoradiographic images of killed ^{35}S labelled *E coli* 25 ×

counters is small Statistical treatment of the logarithmic values of the data showed that the correlation coefficient between two observers was r = 0 996 The deviation from the mean of the individual count only occasionally exceeded a few hundreds of the logarithmic number

The similarity in results obtained by the two independent observers and the high precision shows that the counting method is suitable for routine analysis However a short probation period was required before the autoradiographic images of bacteria could be easily distinguished by workers not familiar with the technique

Experiments were performed to determine the presence of dots on autoradiograms prepared from filters through which lung preparations not containing radioactive bacteria had been filtered The number of such background dots was determined on 35 autoradiograms The filters for these autoradiograms were prepared at the same time as experiments where radioactive bacteria were used

In table 2 the distribution of the background dots is presented as cumulative relative frequencies It is seen for instance that on 6% of the autoradiograms from an experiment performed under the present conditions the background value can be expected to be 14—15 dots

The background dots found on autoradiograms from filters through

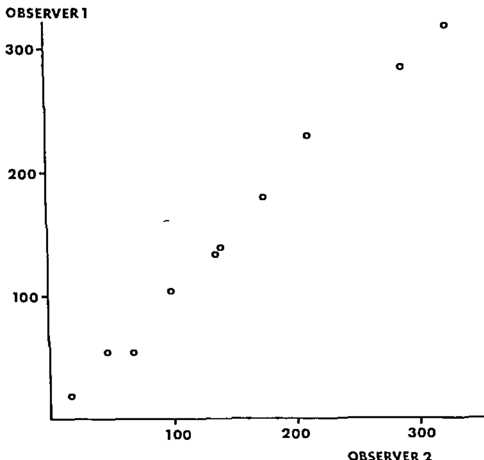


Figure 5 Comparison between countings of radioactive *E coli* images on autoradiograms performed by two independent observers Each value represents the mean of two countings

which preparations not containing radioactive bacteria were filtered are probably of different origin Some might be caused by radioactive bacteria present on the filtering equipment in spite of the rigorous rinsing carried out after each filtration Others might be due to background radiation and others again might be artifacts in the film grain layer itself due to the manufacturing process

The number of background dots on the autoradiograms found in these series of experiments agreed well with the value reported by Berlin and Rylander (1963) They pointed out that if the background dots are studied under higher magnification some of them can be recognized as conglomerates of film grains This procedure was found to increase the sensitivity of the

Table 2

Bacteria like autoradiographic images on control autoradiograms

Dots	Films	P (> x)
0—1	11	100
2—3	10	69
4—5	7	40
6—7	0	20
8—9	3	20
10—11	1	11
12—13	1	9
14—15	2	6
16≤	0	0

method but it also rendered the counting rather tedious Increased exposure time with a resulting larger autoradiographic image is another way to obtain a better differentiation between background dots and the image of the particles under consideration

As a safeguard background values were determined in all experiments and control filters were included in all series The number of radioactive bacteria in the experiments was generally well above the background values and the latter did not interfere significantly with the results

To examine if the reduction in the number of dots on the autoradiograms could be influenced by extraction of radioactivity from the bacteria or disintegration of bacteria the killed radioactive bacteria were suspended in a 5% solution of lysozyme Autoradiographic determinations of the number of bacteria present were made immediately and after 5 hours incubation at 37° C No time effect could be demonstrated on the number of radioactive bacteria found

Although this experiment was partly designed to see within what limits the bacteria remained intact the conditions in the lung *in vivo* are obviously different and the bacteria might be subject to other lytic factors Lysozyme is considered however the most important factor for the lysis of bacteria and the strong resistance to this enzyme, as shown by the results from this experiment indicates that disintegration of the particles used in this experiment plays only a negligible role in the time period in question

Table 3

Number of killed, radioactive (R) and viable (V) E coli found in homogenates of trachea and lower right lobe of lung immediately after exposure

Experiment	Trachea R	Trachea V	Trachea R/V	Lunglobe R	Lunglobe V	Lunglobe R/V
I	65	8	8 1	37	5	7 4
II	77	3	25 7	59	5	11 8
III	109	7	15 6	62	6	10 3
IV	27	3	9 0	81	3	27 0
V	179	5	35 8	96	4	24 0
VI	92	7	13 1	117	6	19 5
VII	59	6	9 8	152	7	21 7
VIII	18	1	18 0	137	7	19 6
IX	17	1	17 0	106	5	21 2
Mean			16 9			18 1

These conclusions agree with earlier reports which were reviewed by Salton (1964) who concluded that gram negative bacteria are very resistant to lysis effected by lysozyme

In order to determine whether the killed radioactive and the viable *E coli* were equally distributed in the lung animals were exposed to the mixed aerosol and the number of the respective cell types determined in trachea grindings and grindings of the lower right lobe The results are found in table 3 The mean from the individual experiments suggests that the distribution of killed radioactive *E coli* and viable *E coli* was almost equal in the trachea and in the lobe of the lung

4 6 Treatment of data

The mean of the value obtained for particles and bacteria from two dilution series of the different preparations was calculated for each animal For each group (one experiment) where animals were killed at various times after exposure this mean value per animal per time interval was expressed as the percentage of the value obtained from the animal examined immediately after exposure

For every time interval the mean and the standard deviation for the per cent units from the different experiments was calculated. The statistical significance of differences between means at various time intervals or between treated animals and control animals at the same time interval was calculated using the student t analysis.

STUDIES ON NORMAL ANIMALS

1 GENERAL CONSIDERATIONS

Experiments were performed on normal guinea pigs to study the reduction of particles and viable bacteria during the first hours after exposure The mechanical elimination of viable *E coli* bacteria was determined in normal animals for various periods during the first hours after exposure and the state of bacteria in the mucus of the trachea, i e if they are inside or outside phago cytes was investigated Finally, the relative importance of the mechanical elimination in the reduction of viable bacteria was determined

2 REDUCTION OF PARTICLES AND VIABLE BACTERIA

The reduction in the number of killed, radioactive *E coli* found in normal animals is illustrated in figure 6 (upper curve) The value at each time interval represents the mean from 20 animals A total of 100 animals were included in this study

It is seen in the figure that the particle reduction was somewhat higher from 0 to 1 and 1 to 2 hours than from 2 to 3 and 3 to 5 hours after which time 50 % of the original number was found in the lungs The standard deviation of the individual values at the various times amounted to ± 14—20 percent units

The reduction of viable bacteria is illustrated in figure 6 (lower curve) As before the value at each time interval represents the mean of determinations on 20 animals

It is seen in the figure that the reduction was larger between 0 and 3 hours after exposure than 3 and 5 hours 16 % of the viable bacteria were recovered after 5 hours The standard deviation of the number of viable bacteria found at the various times amounted to ± 8—27 percent units

If the particle reduction curve is compared to the viable bacteria reduction curve it is seen that the curves are different This difference was found to be almost statistically significant 2 hours after exposure (0 05 > p > 0 01) and significant at 3 and 5 hours after exposure (p < 0 001)

The present study was not aimed at examining the different components of the bactericidal mechanism of the lung However experiments were performed with filter papers impregnated with tracheal mucus and the inhibitory effect on the growth of *E coli* on agar plates was studied An inhibitory activity which

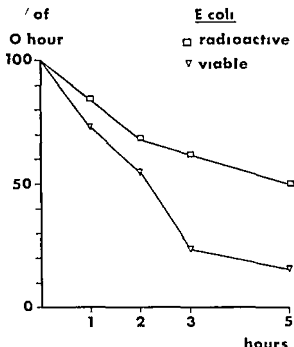


Figure 6 Killed radioactive (□) and viable (▽) *E coli* in lung homogenates various times after exposure Each value represents the mean from 20 animals

was destroyed by heating to 60° C could be demonstrated Although this experiment is only preliminary and without quantitative value, it indicates that the mucus of the guinea pig trachea has a bactericidal activity on the viable *E coli* used

3 MECHANICAL ELIMINATION OF VIABLE BACTERIA

3 1 Flow efficiency (f) determinations

The number of killed radioactive *E coli* found in the trachea and in the lung at various times after the exposure is found in table 4 which reports the findings from 46 animals The value for the number of particles in the lung includes the particles in the trachea

It is seen that a reduction in number took place in the trachea This reduction was larger between 0 and 1 hour after exposure than between 1 and 2

Table 4

Radioactive E coli found in trachea sections (T) and whole lungs (L) various times after exposure The figures given are the mean of two determinations from 1 ml of dual ten fold dilution series of the original preparation (T = 1 dilution, L = 2 dilutions)

	Trachea					Lung				
Experiment	0	½	1	1½	2	0	½	1	1½	2
I	61	186	40	15	32	365	403	221	194	257
II	87	52	26	25	11	421	300	323	332	159
III	63	67	37	26	16	272	314	279	226	252
IV	62	79	86	14	18	293	301	333	155	140
V	64	113	39	13	21	445	334	217	207	225
VI	120	16	5	20	7	281	180	205	165	169
VII	26	36	2	17	69	316	359	328	342	269
VIII	73	24	7	89	29	255	227	320	341	307
IX	50	26	38	—	—	250	261	252	—	—
X	72	14	4	—	—	253	161	204	—	—
Mean	68	61	28	27	25	315	284	268	245	223

hours The particle reduction in the whole lung found in this experiment was closely parallel to that illustrated in figure 6

From the figures in table 4 the flow efficiency f was calculated using equation B 2 (page 27) The f values are reported in table 5

Table 5

Flow efficiency values (f) in trachea of normal guinea pigs

Period after exposure hours	0—½	½—1	1—1½	1½—2
f	4 8	3 6	6 8	8 5

Table 6

Number of viable E coli present in flushings of the inside of trachea sections (T) and homogenates of corresponding lung (V_L) at various times after the exposure The figures given are the means of two determinations from 0 5 ml of an undiluted preparation (T) or two ten fold dilution series (V_L)

	T				V_L			
Experiment	0	½	1	2	0	½	1	2
I	174	159	2	14	149	48	82	28
II	243	49	120	2	156	163	178	51
III	112	16	19	2	158	135	90	72
IV	28	148	16	11	105	14[illegible]	111	29
V	113	54	1	43	163	158	93	47
VI	97	53	17	17	127	80	100	73
VII	71	22	10	54	94	96	83	47
VIII	397	88	124	4	167	144	131	77
IX	113	70	16	2	220	134	111	59
X	245	25	44	2	189	180	133	58
XI	160	12	28	5	143	162	88	40
XII	106	67	76	36	228	195	146	49
XIII	20	190	157	24	156	182	174	83
XIV	143	34	188	45	86	73	52	24
XV	120	79	36	—	100	72	86	61
XVI	131	114	407	85	126	110	135	72
XVII	207	109	12	43	141	146	93	56
XVIII	87	804	49	35	117	166	38	62
XIX	589	135	5	222	201	143	114	80
XX	120	26	14	2	77	90	76	25
Mean	164	113	67	34	145	131	106	55

3 2 V_T determinations

The number of viable bacteria present in washings of the inside of trachea sections is reported in table 6 Measurements were performed simultaneously on the number of bacteria present in the lung It is seen that the number of bacteria in the trachea decreased fairly rapidly the number found 2 hours

Table 7

Number of viable E coli (V) in trachea washings before/after saponine treatment

	Hours after exposure					
	0		1		2	
Experiment	V	% increase	V	% increase	V	% increase
I	108/206	48	62/208	70	9/14	36
II	25/18	—28	6/4	—33	21/8	—62
III	10/40	75	17/34	50	0/0	—
IV	118/150	21	19/24	21	11/16	31
V	123/108	—12	14/82	83	1/0	—
VI	117/130	10	55/56	2	44/30	—32
VII	33/123	73	11/6	—55	9/14	36
VIII	153/160	4	15/12	20	0/0	—
IX	139/144	3	106/108	2	14/22	36
X	194/194	0	38/172	78	32/157	80
Mean		19		24		13

after exposure was about 20 % of the number present immediately after exposure

The reduction of viable bacteria in the whole lung found in this experiment closely paralleled the reduction reported in figure 6

The number of viable bacteria found in tracheal washings before and after addition of saponine is found in table 7 It is seen that an increase of around 20 % took place after addition of saponine

3 3 Calculation of m

From the values in table 5 and 6 the mechanical elimination of viable bacteria (V_T) was calculated and the relative contribution of the mechanical elimination to the reduction of viable bacteria from the lung was calculated using equation A 3 (page 24) The values for m are found in table 8 The calculations of the different m values are found in table 8—addendum in the figure table section (page 84)

It is seen in table 8 that the mechanical elimination of viable E coli amount

Table 8

Mechanical elimination of viable E coli expressed as per cent of reduction of viable bacteria (m) for various periods after exposure

Period after exposure hours	0—1/2	1/2—1	1—2
m	48	13	8

ed to 48 % of the total reduction of viable organisms during the first 1/2 hour after exposure and decreased to 13 % between 1/2 and 1 hour The value be tween 1 and 2 hours was 8 %

4 COMMENTS

4 1 Particles

The general course of the particle reduction curve obtained with radioactive *E coli* is in agreement with findings from earlier authors who have applied other techniques to measure the reduction of particles from the lungs (LaBelle and Brieger 1961)

The results show that about 30 % of particles in the size range of 2 μ which were deposited in the guinea pig lungs under the prevailing conditions were mechanically eliminated from the lung within 2 hours after exposure A 50 % reduction was found after 5 hours This result is in general accordance with the work by Palm et al (1956) who reported almost complete upper respiratory clearance of 1 5 μ particles in less than 6 hours from guinea pig lungs and Harper and Morton (1953) who found a 40—73 % reduction in the number of spores from guinea pig lungs within 5 hours Holma (1967) reported a 50 % reduction within 2 3 hours for 3 μ polystyrene particles from rabbit lungs

Naturally it would have been desirable to compare the loss of particles by mucous elimination with the number of particles found in the stomach at various times after exposure A method for such measurements has been described by Spritzler et al (1967) where the particles were collected in oesophagal fistulae in rats after intratracheal injections

Attempts were made to use this technique in the present experiments using either oesophagal fistulae or measuring the number of particles in the stomach

However they were all unsuccessful due either to technical reasons or to heavy contamination of particles from the nose and mouth which interfered with the measurements Since intratracheal injection and the preparation of an oesophagal fistula represents a major interference in the animal it was decided to avoid these procedures and continue with these studies in the way described in the section on methods carefully checking all other ways in which the particles could disappear from the lung

4 2 Viable bacteria

In general the curve describing the reduction of viable *E coli* from the lung as well as the absence of viable organisms in other organs of the body at various times after exposure is in agreement with the findings of other authors who have used non pathogenic bacteria (Laurenzi et al 1964, Jackson et al 1967) Also the finding that mucus has bactericidal properties is in agreement with earlier reports (Dold 1943)

4 3 Mechanical elimination of viable bacteria

The flow efficiency values reported here cannot be considered as equivalent to the flow rate values reported by other authors (e g Dalhamn 1956) who measured the transport rate of particles in the trachea The f values represent a quantitative measurement of the transport capacity of the whole tracheal tube whereas the particle transport rates are valid only for the particular stream measured This difference in the property measured accounts for the difference in results between Dalhamn who obtained values around 14 mm/min and the results presented here which yield 1 6—2 8 mm/min if the f values are transformed into flow rates

This difference in results also might be explained by differences in experimental procedure Particle transport rates were measured usually with the aid of tracheostomy or on a section of trachea *in vitro* which might influence the mucous flow whereas the present experiments were performed on animals which were intact during the period of the measurement In support for the latter explanation are results reported by Carson et al (1966) who found mucous transport rates of 0 3 mm/min in almost intact cat tracheas

The increase in flow efficiency between 1 and 1 1/2 and 1 1/2 and 2 hours after exposure has not been investigated further Before it can be stated with certainty that this increase is relative to the aerosol exposure itself experiments will have to be performed to determine the influence of handling the animals including the process of putting them into the exposure chamber the environment after exposure and other factors which might be responsible for the observed alteration

The m values found for the various time periods indicate that "m" is an exponential rather than a linear function of time. An improved accuracy will therefore be obtained if the m values are calculated for very short time periods. For the purpose of the present investigations, however, it was considered satisfactory to use half hourly time intervals.

The change in "m" values from 48 % at 0—30 minutes to 18 % at 30—60 minutes suggests that the mechanical elimination of viable bacteria is of greatest importance in the initial postexposure period. This result appears contradictory to the conclusions by Green and Kass (1964 a) who suggested that the bactericidal action of the broncho pulmonary tree was due primarily to the phagocytic activity of the alveolar macrophages and that the action of the mucociliary stream may be related largely to the transport from the lung of phagocytes containing material of bacterial origin.

The results from the present investigation agree that the mucous transport was responsible for only a small part of the total reduction of viable organisms as measured over several hours following exposure. However, the mucous transport was found to be of great significance immediately after exposure and a decrease in transport capacity at that time might well influence the number of bacteria present in the lung at 1 hour after exposure when multiplication of bacteria might commence.

The reason for this discrepancy of results on the relative importance of phagocytosis vs mucous transport has not been subject to experimental analysis. It is probable however that the different experimental techniques may be partly responsible. In the technique used by Green and Kass the lung preparation did not include the major part of the trachea. Bacteria present in this part of the respiratory tree are primarily subject to mechanical removal and if they are not included in the preparation, the relative role of the mechanical elimination is underestimated. In the present experiment the preparations included the trachea as it in studies of this type seems more appropriate from a functional point of view to include the whole respiratory organ from just below the larynx.

The general conclusions concerning the relative role of mechanical elimination of viable bacteria were not influenced by the recorded variations in the flow efficiency values which have been used for the calculations of "m".

The low increase in the number of bacteria found in trachea washings after saponine treatment indicates that most of the viable *E. coli* eliminated from the lung via the mucous transport are not to be found inside phagocytes. These conclusions concerning the state of bacteria in the mucus are in agreement with the findings of Cralley (1942) who detected viable bacteria in the trachea at various times after the exposure using a technique which did not

affect the phagocytes They appear, however, to be contradictory to the con *clusions drawn by Green and Kass (1964 a) who observed that most of the* bacteria in lung sections were inside phagocytic cells very shortly after expo sure However, their observations were made on histological sections of the lung which is not a procedure suitable for quantitative interpretations The possibility also exists that bacteria lying free in or on the mucus might be removed during the preparation of the sections

It cannot be ruled out that the difference in the number of viable bacteria found before and after saponine treatment may be partly due to the dissolu tion of bacterial aggregates in the mucus, although the number of such aggre gations should be small because the preparations were always vigorously shaken This possible error, nevertheless would not influence the above con clusion concerning the presence of free bacteria in the mucus

STUDIES ON EXPOSED ANIMALS

1 GENERAL CONSIDERATIONS

Any variation from the normal in the number of viable bacteria found in the lung at various times after deposition can be due to a change in the bactericidal capacity of the lung. Thus, an increased number of viable bacteria may result from a decreased bactericidal capacity and vice versa.

In cases where the mechanical elimination of viable bacteria from lung is of importance the condition above is not always true. A decreased mucous transport may be the cause of an increased number of viable bacteria in the lung without any change in the bactericidal capacity. Similarly it is possible that an increased activity of one system could mask a decreased activity in the other.

To understand the cause of an observed variation in the reduction of viable bacteria the number of insoluble particles and viable bacteria in the

Table 9

Evaluation of alterations in the bactericidal capacity of the lung by determining the number of particles and viable bacteria present in lung at a given time after deposition (Bactericidal capacity o = normal + = increased – = decreased)

Particles compared to normal	Viable bacteria compared to normal		
	More	Equal	Fewer
More	0 + –	+ 0	+
Equal	–	0	+
Fewer	–	0 –	+ – 0

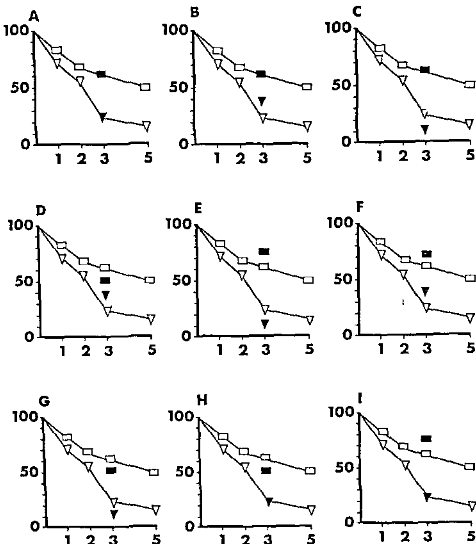


Figure 7 Representative results from experiments where the number of particles (upper curve) and viable bacteria (lower curve) found in lungs of treated animals (black symbols) has been altered as compared to normal values See text for further discussion

lung has to be determined at various times after deposition In table 9 the possible alterations in the bactericidal capacity of the lung are presented for all combinations of results from such measurements Representative results applicable to the different combinations in the table are found in figures 7 a—i

It is seen in table 9 that in cases where the number of particles found in the lung does not vary from the norm the measurement of viable bacteria will give

a clearcut answer as to the capacity of the bactericidal mechanism Such results are illustrated in figures 7 a—c

In cases where the number of particles and viable bacteria change in an inverse manner i e fewer particles and more bacteria or more particles and fewer bacteria, the functioning of the bactericidal mechanism is easily evaluated also Such results are illustrated in figures 7 d—e

In cases where the number of particles and viable bacteria change in a direct manner, both either increase or decrease or if the number of particles changes but the number of viable bacteria remains unchanged, the change in the bactericidal mechanism cannot be determined directly These cases are illustrated in figures 7 f—i

In case 7 f the increase in the number of particles found in the lung after 3 hours is due to a decrease in the mucous transport The increased number of viable bacteria found could be due either to a decreased capacity of the bactericidal mechanism or to the decrease in the mucous transport in which case the bactericidal capacity might have remained unchanged If the mechanical elimination of viable bacteria is the most important component in the total reduction of viable bacteria (m→100) it is also possible that the decreased transport could mask an increase in the bactericidal capacity, resulting in an increased number of viable bacteria The above principal reasoning is applicable also to case 7 g

In case 7 h where no alteration in the number of viable bacteria occurred the decrease in the number of particles found indicates an increase in mucous transport This increased mucous transport might have masked a decrease in the bactericidal capacity by removing a larger number of viable bacteria than normally The same type of reasoning applies to case 7 i

When experimental results such as those presented in case 7 f—i are found the functioning of the bactericidal mechanism can be estimated if the insoluble particles and the viable bacteria used have equal deposit and mechanical elimination characteristics A change in the particle reduction will reflect a corresponding change of the mechanical component responsible for the reduction of viable bacteria (equation A 2 page 24) The remaining change in the reduction of viable bacteria will be due to a change in the bactericidal capacity of the lung

The occurrence of cases 7 f—i will be determined by the relative importance of the mechanical elimination of viable bacteria i e the value of "m In the present experiments wh e *E coli* was studied the m value was of significance only 1/2 and 1 hou after exposure When the measurements were made 3

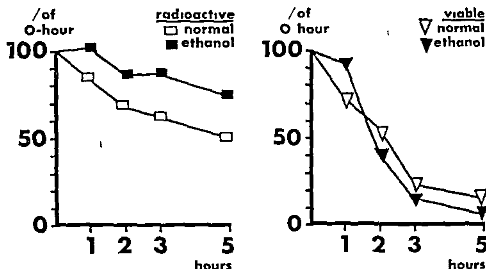


Figure 8 Killed radioactive and viable *E coli* found in lungs of ethanol treated guinea pigs at various times after exposure (black symbols) compared to normal values (white symbols)

74% of the radioactive bacteria remained in the lungs of the ethanol treated animal as compared to 50 % in the normal

The number of viable bacteria was lower in the ethanol treated group than in the normal at 3 and 5 hours after exposure this difference was statistically significant at 5 hours ($p < 0\ 001$) An increased number was found at 1 hour after exposure—this increase was almost statistically significant ($0\ 05 > p > 0\ 02$)

2 1 3 *Comments*

The results from the ethanol exposure experiment correspond to the hypothetical case illustrated in figure 7 f at 1 hour after exposure and to 7 e at 3 and 5 hours after exposure

The increased number of killed radioactive bacteria found at 3 and 5 hours after exposure shows that the mucous transport had been decreased by the ethanol treatment This conclusion is in agreement with an earlier report by Laurenzi and Guarneri (1966) who observed a decreased flow rate in the trachea of ethanol treated cats

According to Nungester and Klepser (1938) the function of the glottis is impaired in rats subject to ethanol intoxication The occurrence of this effect in the guinea pigs examined here could have caused aspiration of radioactive bacteria from the mouth and thus accounted for the larger number of radioactive bacteria found in the lungs However since most of the bacteria used in

this experiment were of a particle size that favours deposition in the deeper airways with relatively little deposition in the upper respiratory tree an impairment of the glottis is not considered to have influenced the result to any large extent

The increased number of viable bacteria found at 1 hour after exposure when the mechanical elimination of viable bacteria is of importance (table 8) suggests that a reason behind the increase is the reduced mucous transport Control experiments on ethanol exposed animals who were not exposed to bacteria failed to detect any contamination with the normal mouth flora in the lung Consequently, an eventual impairment in the functioning of the glottis was not considered to contribute significantly to the increase in viable bacteria at 1 hour after exposure

The decreased number of viable bacteria found at 3 and 5 hours after exposure shows that the bactericidal capacity of the lung had increased

The results from the present experiments concerning the reduction of viable bacteria are contradictory to those reported by Green and Kass (1965) who found more bacteria in the whole lung of mice after ethanol treatment The reason for this discrepancy is not clear Since the dosage levels used were in the same range it seems likely that differences in the reaction of phagocytes between the two types of animals may be responsible The existence of such species bound differences was reported by Laurenzi and Guarneri (1966)

2 2 Coal dust

2 2 1 *Exposure conditions*

Guinea pigs in a chamber were exposed to ordinary room air containing carbon black particles at an average concentration of 15 mg/m^3 air for 6 hours a day 5 days per week for 3 weeks The aerosol was generated in a plexiglass tube where a fan suspended carbon black lying on the bottom of the tube Larger particles were collected by sedimentation in an adjacent horizontal tube before the coal aerosol entered the exposure chamber About 90 % of the particles sampled from the chamber were around 2 μ in size The particle size was measured on Millipore filters with the aid of a microscope Control animals were exposed to ordinary room air in another chamber

At the end of the exposure period the animals were taken directly from the chambers and were exposed to the mixed *E coli* aerosol under conditions earlier described Pairs of one control animal and one coal dust treated animal were killed immediately and 1 and 3 hours after this exposure Determinations of the number of radioactive and viable bacteria in lung homogenates were made by the procedure described earlier

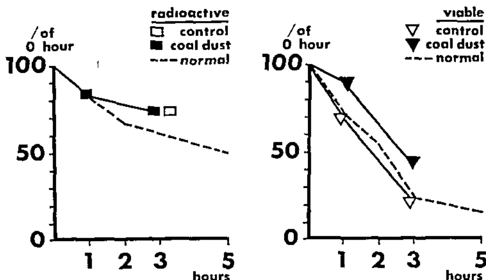


Figure 9 Killed radioactive and viable *E coli* found in lungs of guinea pigs exposed to coal dust 1 and 3 hours after exposure (black symbols) compared to control animals(white symbols) Normal values indicated as dotted lines

2 2 2 *Results*

The number of killed radioactive and viable *E coli* found in lung homogenates is shown in figure 9 The values represent the mean from 15 coal dust treated animals and 10 control animals at each observation time

It is seen that the reduction of radioactive E coli was equal in the treated and in the control animals It should be noted however that the 3 hour value *in the control animals was somewhat higher than the normal value reported* earlier (figure 6 page 42) This difference which has consistently been *found when older animals were examined was almost statistically significant* ($0\ 05 > p > 0\ 02$)

The reduction of viable bacteria in the lungs of coal dust exposed animals was lower than in the control animals at 1 and 3 hours after exposure, the difference after 3 hours was statistically significant ($p < 0\ 001$) The values for the control animals were not different from that of the normal animals examined earlier

2 2 3 *Comments*

The findings in the coal exposure experiments correspond to the hypothetical case illustrated in figure 7 b

The similarity in the number of radioactive bacteria present in control and coal dust exposed animals indicates that the mucous elimination was not affected by the latter exposure

The lower reduction of viable bacteria in the coal dust exposed animals suggests that the bactericidal activity of the lung had been impaired In view of the finding from histological sections taken from exposed animals, where large amounts of coal particles were found inside phagocytes it seems reasonable to assume that the lower reduction of viable bacteria was due to a decreased capacity of the phagocytes to inactivate the bacteria

The conclusions drawn from this experiment agree with numerous earlier findings that a change in the defence mechanism of the lung against bacteria can occur after exposure to dust Many of these studies have been made using atypical tubercle bacteria (Policard and Dufourt 1937 and Tacquet et al 1966)

Several reports however, failed to find any influence of dust exposure (Baetjer 1944 1947 1951 and Vintinner 1951) In these studies the response to an intrabronchial injection of pneumococci was measured in control rats and rats exposed to various kinds of dust In general the investigations showed that exposure to dust did not increase the susceptibility of the rats to lobar pneumonia However in contrast to the studies with tubercle bacteria the pneumococci produced a very severe effect which also occurred excessively among control animals Since pneumococci are less readily phagocytized changes in the functioning of the phagocytes would be difficult to detect with this experimental model Results from *in vitro* experiments with phagocytes from dust exposed animals (Voisin et al 1963) indicate that changes which might exist in phagocytes after dust exposure can be difficult to detect

2 3 Cigarette smoke

2 3 1 *Exposure conditions*

Guinea pigs were exposed to cigarette smoke in an exposure chamber which allowed fresh smoke to be blown directly on their noses The smoke was generated by sucking 35 ml of air through lighted cigarettes for a two second period once every minute Fresh air was rapidly introduced into the chamber between puffs in order to obtain intermittent exposure as present under human conditions Nine to ten puffs were obtained from each cigarette and the animals were exposed to smoke from 1 cigarette daily for 5 days thereafter they were exposed to smoke from 2 cigarettes in the morning and 2 in the afternoon for 10 days Compared to the control animals the smoke exposed

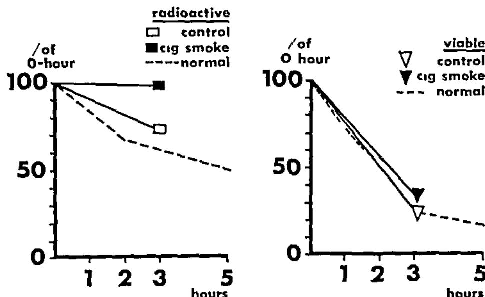


Figure 10 Killed radioactive and viable *E coli* found in lungs of guinea pigs exposed to cigarette smoke 3 hours after exposure (black symbols) compared to control animals (white symbols) Normal values indicated as dotted lines

animals increased in weight only slightly during the duration of the experiment No obvious symptoms of respiratory disease were observed

The day after the last smoke exposure the animals were exposed to the mixed *E coli* aerosol and the number of killed radioactive and viable bacteria was determined immediately and 3 hours after the exposure

To compare deposition characteristics of the radioactive bacteria in the smoke exposed animals and in the controls, the number of radioactive bacteria in a section of the trachea and in the lower right lobe of the lung was determined immediately after exposure The ratio between the trachea and lobe values was calculated and the value from the smoke exposed animals compared to that of the controls The control animals in this experiment were the same as in the coal dust experiment

2-3 2 *Results*

The number of killed radioactive and viable *E coli* found in lung homogenates of cigarette smoke exposed animals and control animals is illustrated in figure 10 The values represent the mean of 12 smoke exposed animals and 10 control animals at each observation time

The number of radioactive bacteria in the smoke exposed was foun

to be higher than in the controls, this difference was almost statistically significant ($0.05 > p > 0.02$)

The number of viable bacteria in the smoke exposed animals was slightly higher than in the controls, however this difference was not statistically significant

The trachea/lobe ratio for radioactive bacteria immediately after exposure found in the smoke exposed animals was not significantly different from that of the controls

2 2 3 *Comments*

The results from the smoke exposure experiment correspond to the hypothetical case illustrated in figure 7 1

The larger number of radioactive bacteria found in the lungs of cigarette smoke exposed animals suggests that the mucous transport had been decreased This conclusion agrees with the findings of Carson et al (1966) who observed a decreased mucous flow in the trachea of cats exposed to cigarette smoke

Bair and Dilley (1966) and LaBelle et al (1966) on the other hand were not able to detect an effect on the pulmonary clearance of inhaled inert particles in animals exposed to cigarette smoke In these experiments pulmonary clearance was measured by the body scanning technique However the measurements in the first mentioned experiments were not conducted during the first hours after exposure but at daily intervals The latter experiments were acute smoke exposures Therefore it appears probable that changes in the initial mucous transport which were found in the present experiments after chronic exposure were not detected

GENERAL DISCUSSION

1 METHODS

Radioactive labelling of bacteria has been used by several authors to study the distribution of bacteria in the body of animals (Harper and Morton 1962 Green and Goldstein 1966)/

Animals were exposed to an aerosol of these microorganisms and the amount of radioactivity present in the lung was measured at various times after exposure The decrease in radioactivity was considered to represent the elimination of the bacterial particles from the lung Although measurements of the total amount of radioactivity is a satisfactory method, the sensitivity is increased and comparison between the viable count values and the particle reduction values facilitated if single particles are counted as in the autoradiographic method described here

Autoradiographic techniques have been used to trace radioactively labelled bacteria in previous experiments (Ely 1942 Pasquier 1965) Some general drawbacks using autoradiographic tracer techniques when working with bacteria have been summarized by Bonventre and Imhoff (1965) They pointed out that the intensity of the radioactivity observed and the number of viable bacteria were not always proportional Also when the labelled organisms began to multiply, which could occur within a few hours after deposition (Green and Kass 1964 b), the dilution of the radioactivity per bacterium might result in an autoradiographic distribution pattern which is not consistent with the number of bacteria present

The above drawbacks were avoided with the procedure used in the present investigation Since killed bacteria were used no multiplication occurred and moreover the radioactive tracer was bound so firmly that the amount of radioactivity per particle would not decrease except upon disintegration of the particle The number of autoradiographic images found therefore, should represent the number of particles present The quantitative evaluation of the number of radioactive bacteria in homogenates of whole lungs by counting the images on autoradiograms has been shown to be reasonably accurate and is probably a more sensitive method than densitometry readings suggested by Bonventre and Imhoff

The autoradiographic technique described here readily lends itself to the study of radioactively labelled bacteria of other species or other kinds of

particles of varying sizes, provided that it is shown in control experiments that the radioactive tracer within the time of the experiment is not liberated from the particle to such an extent that an autoradiographic image will not occur

In the present experiments non pathogenic viable bacteria were used to measure alterations in the functioning of the bactericidal mechanisms It seems probable that conclusions concerning the effect of various types of treatment are valid also for pathogenic organisms However if an experimental model with pathogenic bacteria had been used where the pathogenic effect caused by the bacteria was measured the changes due to a certain treatment might have been more difficult to detect This difficulty could be due to a variation in the susceptibility to infection of the control animals or to the fact that pathogenic bacteria are less readily destroyed by phagocytes Thus even a fairly large change in the functioning capacity of the phagocytes might not lead to any measurable change in the number of free, viable bacteria and a difference in incidence of the measured effect between normal and treated animals would not be observed Therefore the use of non pathogens in this type of study appears to be preferable

Although the methods used here offer several advantages some drawbacks must be borne in mind Since one animal has to be killed for each determination the variation between the animals within a group may be quite large Consequently a fairly large number of animals has to be examined when the effect of a specific agent is to be evaluated Variations in the number of bacteria originally deposited in the animals and individual elimination rates contribute to the experimental error Experimental models where the elimination in the same animal can be measured both before and after the treatment (Holma 1967) lessen the effect of these errors and might be more sensitive under certain circumstances On the other hand the increased cost and labour involved when large groups of animals are used can be compensated for by the low cost of the equipment required for the present techniques

2 RESULTS

The results presented here have shown that the mechanical elimination of viable *E coli* is of greatest importance during the first hour after exposure but is less significant for the reduction of viable bacteria at 2 hours and later after exposure

This relation between the mechanical elimination and the bactericidal mechanism is valid only for the non pathogenic *E coli* under the conditions present in this experiment and for the normal animals A measurement of the importance of the mechanical elimination should be performed for each

type of bacterium and for each type of experimental situation before conclusions concerning the relative role of the mechanical elimination can be drawn

It must be borne in mind that the deposit distribution could influence the relation between the mechanical elimination and the bactericidal effect — the more bacteria that deposit in the mucus the more can be expected to be eliminated via the trachea Hence, the size of different species of bacteria might influence this relation

With mono disperse aerosols of bacteria, where the particles have a size of 1—2 μ, the deposition is likely to occur predominantly in the peripheral regions of the lung which of course decreases the relative importance of the mechanical elimination at least for non pathogenic bacteria On the other hand realistic environmental conditions probably involve exposure to bacteria of widely varying sizes in which case the larger particles will deposit further up in the lung This type of exposure will increase the importance of the mucous transport

In areas where the normal boundary layer of the inhaled air is disturbed i e just below the larynx and at bronchial branchings (Ingelstedt and Tore malm 1961) conditions are favourable for the deposition of particles In such areas the mucous transport is also of great importance

The relative role of the mucous transport could be larger for bacteria which are less readily rendered non viable by phagocytes than for non pathogens of equal size Further studies should be undertaken to elucidate this problem

Bacteria which are in contact with the mucus and either killed or prevented from outgrowth by its bactericidal agents are simultaneously removed mechani cally from the lung by the mucus within a fairly short time It is reasonable, therefore to assume that the bactericidal activity of the mucus under normal circumstances plays a secondary role in the defence as compared to the mechanical transportation at least for the type of bacterium studied in this work Thus the activity of the phagocytes is probably the most important of the two bactericidal mechanisms discussed here However, the relative importance of the bactericidal effect of the mucus and the phagocytes could be different when the defence against pathogenic bacteria is concerned

The results from the exposure experiments further illustrate that the inter action and relative importance of the individual defence mechanisms involved must be measured in every experimental situation before any conclusion con cerning a specific effect of a certain treatment can be drawn

If in the ethanol and coal exposed animals the reduction of viable bacteria had been measured only the observed difference between normal and exposed animals could have been due to both a change in the mucous transport and a change in the bacteri idal activity of the lung Knowledge of the relative importance of the m anical elimination (m) was essential here If on the

other hand the test aerosol had contained particles only, the effect of the exposure on the bactericidal activity would have remained undetected

The decrease of the functioning of the mucous transport mechanism observed in the ethanol and in the smoke exposed animals could be due either to a change of physical properties of the mucus or an alteration of the ciliary beat (Rylander 1966) Some recent studies indicate that changes in the composition or amount of mucus rather than an effect on the cilia themselves are more important following exposure to agents in concentrations comparable to those encountered in human exposures (Dalhamn and Reid 1966) The development of methods to measure the physical properties of mucus is therefore a most important research goal

The primary aim of this work has been to elucidate the functions of the pulmonary defence mechanisms against bacteria by developing methods suitable for the measurement of the two major mechanisms involved It is clear however that the effect of coal dust on the phagocytes and the effect of cigarette smoke on the mucous transport is also of importance for the pulmonary elimination of other kinds of particles than bacteria A decrease in the mucous flow rate apart from allowing viable bacteria which lie free on the mucus to multiply and cause damage before they are removed from the lung also will allow toxic substances which have deposited on the mucus to remain in contact with the epithelium for a longer time with increasing risk for damages It cannot be ruled out that a reduced capacity of the phagocytes to kill bacteria also reflects a decreased capacity to incorporate and remove inorganic particles from the lung

Thus the methods employed here for studying the elimination of bacteria from the lung can be of value in general studies of the defence mechanisms of the lung and how they are affected by different physiological conditions and environmental agents such as pharmaceutical products and air pollution

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total reduction of viable bacteria between 0 and 1/2 hour after exposure This figure decreased to 13 % between 1/2 and 1 hour and 8 % between 1 and 2 hours It was shown also that most of the viable bacteria eliminated via the trachea were situated outside phagocytes

Experiments were performed on guinea pigs which were inoculated intra peritoneally with ethanol after the bacteria exposure and guinea pigs which three weeks prior to the bacteria exposure had been exposed to coal dust or fresh cigarette smoke in inhalation chambers

Following ethanol treatment more killed radioactive bacteria were found in the lungs of the treated animals than controls which indicated that the mucous transport was impaired by ethanol Initially more viable bacteria were found in the lungs of ethanol treated animals but this number decreased below the normal 3—5 hours after the exposure These results indicate that ethanol caused a reduction in the mechanical elimination of viable bacteria via the mucous transport at a time when this process was relatively impor tant This effect was later compensated by the increased capacity of the bac tericidal mechanisms of the lung

The results from the coal dust exposure experiments showed that the reduc tion of killed radioactive bacteria was equal to control values indicating that the mucous flow was not affected An increased number of viable bacteria found in the coal dust exposed animals indicates that the efficiency of the bac tericidal mechanisms had been impaired

In the cigarette smoke exposed animals the reduction of killed, radioactive bacteria was lower than the controls which was interpreted as an effect on the mucous flow No significant difference in the reduction of viable bacteria was detected

Although the results reported here are valid only for the prevailing experi mental conditions they show that the relative importance of the different elim ination mechanisms has to be measured for every experimental situation especially when the effect of a certain type of treatment is to be evaluated For such studies the methods developed here seem to be suitable

The investigations were performed primarily to study the defence mecha nisms of the lung towards inhaled bacteria but the experimental system devel oped also appears suitable for general studies of the ability of various agents e g air pollutants and pharmacological preparations to influence the pulmo nary defence mechanisms against inhaled bacteria and other particles

SOMMAIRE

Cette recherche a porté sur l'étude du pouvoir du poumon de cobaye d'éliminer les bactéries non pathogéniques inhalées, une attention spéciale étant accordée au rôle relatif de l'élimination mécanique de bactéries viables pendant les premières heures après l'exposition.

Des méthodes furent mises au point pour mesurer l'élimination mécanique de bactéries viables via le système de transport de mucus et l'activité bactéricide de tout le poumon. Ces activités furent étudiées sur des animaux normaux et sur des animaux exposés à l'éthanol, la poussière de charbon et la fumée de cigarette.

La proportion de bactéries viables éliminées mécaniquement fut évaluée par comparaison du nombre de bactéries viables éliminées via la trachée artère avec la réduction des bactéries viables provenant de la totalité du poumon.

Les cobayes ont été exposés à un aérosol contenant des bactéries *E. Coli* viables et des spores tués des mêmes espèces marqués avec le radioisotope ^{35}S. Le nombre de bactéries viables et radioactives présentes dans le poumon a été déterminé dans les animaux tués de nombreuses fois après l'exposition et comparé au nombre présent immédiatement après l'exposition.

Une technique bactériologique conventionnelle a été employée pour déterminer le nombre de bactéries viables présentes dans des unités homogènes du poumon ou des parties de ce dernier.

Le nombre de bactéries radioactives a été déterminé à l'aide d'une technique auto-radiographique par filtrage d'échantillons de parties homogènes de poumon à travers un filtre de Millipore reposant sur un film radiographique. Après exposition et développement, les bactéries radioactives furent visibles sur le film comme de petits points noirs et comptés avec un agrandissement de 30 fois.

Dans des animaux normaux, le nombre de bactéries radioactives a été réduit d'environ 50 % 5 heures après l'exposition, un chiffre qui concorde avec les résultats d'autres recherches sur l'élimination de particules inertes de même taille (1.5–2.5 μ). La réduction a été plus rapide entre 0 et 2 heures après l'exposition qu'entre 2 et 5 heures.

Le nombre de bactéries viables diminua plus rapidement que celui de bactéries radioactives. 5 heures après l'exposition, environ 16 % des bactéries viables restaient dans le poumon.

L'élimination de bactéries viables via la trachée artère a été étudiée par détermination de l efficacité d écoulement du transport de mucus dans la trachée artère et du nombre de bactéries viables présentes dans la trachée artère à de courts intervalles après l exposition Il a été constaté que l élimination mécanique répond de 48 % de la diminution totale de bactéries viables entre 0 et ½ heure après l exposition Ce chiffre tomba à 13 % entre ½ et 1 heure, et à 8% entre 1 et 2 heures Il a donc été démontré que la majorité des bactéries viables éliminées via la trachée artère se trouvaient en dehors des phagocytes

Des expériences furent effectuées sur des cobayes inoculés intrapéritonéalement avec de l éthanol après exposition aux bactéries et sur des cobayes qui trois semaines avant l exposition aux bactéries ont été exposés à de la poussière de charbon ou de la fumée de cigarette fraîche dans des chambres d inhalation

A la suite de traitement a l éthanol plus de bactéries radioactives tuées ont été trouvées dans les poumons des animaux traités, ce qui indique le transport de mucus a été retardé par l éthanol Au début un nombre plus grand de bactéries viables a été trouvé dans les poumons d animaux traités a l éthanol mais 3—5 heures après l exposition le nombre descendit sous la normale Ces résultats indiquent que l éthanol entraîna une réduction dans l élimination mécanique des bactéries viables via le transport de mucus quand ce processus aurait du être relativement important Cet effet a été compensé ultérieurement par la capacité accrue des mécanismes bactéricides du poumon

Les résultats des expériences d exposition à la poussière de charbon montrèrent que la réduction des bactéries radioactives tuées était égale aux valeurs de contrôle ce qui indique que le flux de mucus n a pas été influencé Le nombre accru de bactéries viables trouvées dans les animaux exposés a la poussière de charbon indique que l efficacité des mécanismes bactéricides a été réduite

Dans les animaux exposés à la fumée de cigarette la réduction des bactéries radioactives tuées a été plus faible que les valeurs de contrôle ce qui a été interprété comme un effet sur le flux de mucus Aucune différence importante ne fut constatée dans la réduction des bactéries viables

Bien que les résultats présentés ici ne soient valables que pour les conditions expérimentales régnantes ils montrent que l importance relative des différents mécanismes d élimination doit être mesurée pour chaque situation expérimentale et particulièrement quand l effet d un certain type de traitement doit être évalué Les méthodes mises au point ici semblent convenir a de telles études

Les recherc[illegible] ont été primairement effectuées pour étudier les mécanismes de défense d[illegible] poumon envers des bactéries inhalées mais le système expéri

mental mis au point semble egalement approprie pour des etudes generales sur le pouvoir de differents agents, par exemple des preparations pharmacologiques et des agents polluant l air, d influencer les mécanismes de défense pulmonaires envers des bacteries et d autres particules inhalées

ВЫВОДЫ

Данная работа имела целью исследование особенности легкого морской свинки связанной с невосприимчивостью к непатологическим видам бактерий полученных через дыхательные пути особое внимание уделялось соответствующим способностям к механическому выделению жизнеспособных бактерий в первые часы после начала эксперимента

Метод разрабатывался путем измерения механического выделения жизнеспособных бактерий через систему слизистых путей и бактерицидной активности всего легкого Опыты велись на обычных животных и на тех что подверглись действию этанола угольной пыли сигаретного дыма

Соотношение жизнеспособных бактерий выделенных механически было определено путем сравнения количеств жизнеспособных бактерий выделенных через трахею и через все легкое

Морские свинки подвергались воздействию аэрозольной смеси живых *Escherichia Coli* и убитых бактерий той же группы известных как ^{35}S Количество жизнеспособных и радиоактивных бактерий находящихся в легком было определено у животных убитых в разное время после вакцинации и сравнено с количеством бактерий у животных убитых сразу же после вакцинации

Для определения количества жизнеспособных бактерий в однородных легких (или их частях) был использован общеизвестный бактериологический метод

Количество радиоактивных бактерий было определено авторадиографическим способом — рентгенографией фильтров Миллипора через которые были профильтрованы образцы легкого После выделения и развития радиоактивные бактерии были представлены в виде черных точек на пленке увеличенной в 30 раз и подсчитаны

У нормальных животных количество радиоактивных бактерий снизилось на 50 % через пять часов что соответствовало результатам других опытов по подсчетам выделения инертных частиц того же размера (1 5—2 5μ) Снижение происходило более быстро между 0 и 2-мя часами после начала эксперимента чем между 2-мя и 5-ью часами

Количество живых бактерий уменьшилось быстрее чем количество радиоактивных Через 5 часов после начала только 16 % живых бактерий оставалось в легких

Выделение живых бактерий через трахею изучалось определением слизистых выделений в трахее и числом живых бактерии находящихся в трахее через короткие промежутки времени после начала. Механические выделения соответствовали 48 % от общего снижения количества живых бактерий между 0 и 1/2 часа после начала. Этот показатель снижается до 13 % между 1/2 и 1 часом и 8 % между 1 и 2 часами. Это доказало также, что большинство живых бактерии выделенных через трахею было найдено вне фагоцитов.

Эксперимент был проведен над морскими свинками, которым была введена внутрибрюшинная инъекция этанола после выделения бактерий и над морскими свинками, которые за три недели до выделения бактерий были подвергнуты действию угольной пыли и сигаретного дыма в ингаляционных камерах.

В течении лечения этанолом было обнаружено, что в легких животных, подвергавшихся лечению было найдено больше радиоактивных бактерии, чем в легких контрольных животных, это свидетельствовало о том, что состояние слизистых оболочек первых было ухудшено этанолом. Первоначально более жизнеспособные бактерии были найдены в легких животных, получивших лечение этанолом, но их число уменьшилось за пределы нормального через 3—5 часов после заражения. Эти результаты показывают, что этанол влечет за собой снижение механического выделения жизнеспособных бактерий через слизистые пути как раз в то время, когда этот процесс особенно важен. Однако позднее этот эффект компенсируется увеличением активности бактерицидного механизма легкого.

Результаты эксперимента с введением угольной пыли подтверждают, что уменьшение количества радиоактивных бактерий соответствует контрольному числу, показывая тем самым, что слизистая оболочка не была подвергнута воздействию этанола. Возросшее количество жизнеспособных бактерий, найденное у животных, подвергавшихся действию угольной пыли показывает, что эффективность бактерицидного механизма снизилась. Уменьшение количества радиоактивных бактерий у животных, подвергнутых действию табачного дыма было меньше, чем у контрольных, это рассматривалось как воздействие на слизистую оболочку. Никакой заметной разницы в уменьшении жизнеспособных бактерий не было отмечено.

Хотя результаты, приведенные здесь, действительны главным образом [illegible] экспериментальных условий, они показывают, что

относительная важность различных очищающих механизмов должна определяться для каждой экспериментальной ситуации особенно когда речь идет о конкретном типе лечения Для подобных опытов предложенные здесь методы кажутся вполне приемлемыми

Данное исследование было проделано главным образом для изучения защитного механизма легких от вдыхаемых бактерий однако разработанная экспериментальная система пригодна и для общего изучения способности различных агентов (таких как — загрязнители воздуха и фармакологические препараты) к действию на легочный защитный механизм против вдыхаемых бактерий и других частиц

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The author

APPENDIX

Equipment	Trade mark Characteristics	Manufacturer
	A Bacteria exposure	
Exposure chamber	Stainless steel polished, plexiglass ports vol 34 liters	Drawings available from author
Bacteria filters	Bicapa 99 Cellulose filters, retention 95 % of DOP aerosol (particle size $^1/_{10}\,\mu$)	Bicapa Stockholm Ö Sweden
Collison atomizer	Capacity 0 35—0 55 ml bacteria suspension/min required air pressure 28 psi	Lit reference Henderson DW (1952)
Compressor	Sprayit 820 2 c 3 3 l³/min at 28 psi	Thomas Ind Inc Sheboygan Wisc USA
Exhaust pump	Wade VP 450 F 12 7 m³/hr atm pressure	J T Wade High Wycombe Bucks England
	B Bacteria culturing	
Medium for radioactive labelling	Modified Anderson s medium MgCl 0 1 g Na_2HPO_4 6 g KH_2PO_4 3 g NaCl 5 g NH_4Cl 1 g glucose 4 g water ad 1000 ml pH 7 sterilize at 110 C 20 min	

	SJS 2 P sterile carrierfree sulphate	Radiochemical Centre Amersham England
	C Bacteria determination	
	Millipore filter type HA 0 45 μ	Millipore Filter Corp Bedford Mass U S A
	Bausch Lomb stereozoom microscope type BVB 73 Magnification plate counts × 14 autoradiographical counts × 30	Bausch and Lomb
Counter	Automatic pen point or foot pedal touch counter	Drawings available from author

FIGURE-TABLES

The values, on which the figures are based, are found in this figure table section under the corresponding figure number The values given here are the number of viable *E coli* found in 0 5 ml of the preparation fluid after one ten fold dilution and the number of radioactive *E coli* found in 1 0 ml of the prep aration fluid after two tenfold dilutions Determinations on dual dilution series were made in all cases

Figure 6—table A

Number of radioactive E coli in lung homogenates various times after exposure

	Hours after exposure				
Experiment	0	1	2	3	5
I	147—122	149—157	76—74	104—103	59—68
II	142—202	169—170	137—136	69—77	51—65
III	189—186	209—200	126—121	100—97	105—99
IV	349—318	220—279	198—214	229—213	115—113
V	363—371	222—234	335—335	162—159	136—151
VI	418—360	245—250	241—239	198—230	192—188
VII	257—262	224—204	190—118	168—174	128—155
VIII	385—362	264—279	108—127	167—147	102—129
IX	330—250	291—291	237—236	204—164	228—222
X	519—477	257—321	274—296	242—244	233—235
XI	367—386	403—375	216—214	289—293	271—255
XII	516—365	398—370	390—390	282—294	261—221
XIII	387—370	375—258	216—212	180—198	210—213
XIV	343—342	306—284	203—192	255—262	214—211
XV	383—414	368—380	277—293	345—352	198—203
XVI	165—177	105—125	158—145	147—146	111—105
XVII	188—197	197—185	220—192	156—122	120—108
XVIII	240—299	192—151	209—176	235—246	153—192
XIX	280—257	280—310	129—128	120—131	92—95
XX	286—279	132—107	175—156	89—99	56—88
Mean per cent	100	84	67	62	50
S D per cent units	—	20	18	17	14

Figure 6—table B

Number of viable E coli in lung homogenates various times after exposure

	Hours after exposure				
Experiment	0	1	2	3	5
I	471—468	647—654	212—200	45—36	100—102
II	590—488	211—279	328—339	123—145	96—86
III	751—690	515—541	219—245	119—151	171—171
IV	437—473	366—395	394—380	165—152	135—137
V	593—683	340—344	389—366	133—133	82—83
VI	443—474	245—273	137—146	328—341	59—79
VII	762—656	258—279	262—289	82—80	58—75
VIII	509—530	473—477	195—202	90— 83	56—67
IX	683—732	405—355	468—460	214—223	232—266
X	791—806	708—672	531—509	60—82	89—85
XI	277—268	234—255	72—61	33—37	50—54
XII	175—183	226—220	71—86	90—92	44—46
XIII	212—189	138—154	147—167	20—23	36—34
XIV	93—110	83—93	70—67	44—29	20—15
XV	217—221	91—89	46—50	11—6	6—14
XVI	542—586	335—298	490—452	158—143	46—37
XVII	680—695	336—333	282—295	208—242	89—91
XVIII	830—831	384—419	475—442	148—141	76—77
XIX	204—190	194—192	202—217	23—25	7—12
XX	318—335	197—245	138—195	63—48	32—28
Mean per cent	100	73	53	23	16
S D per cent units	—	27	23	16	8

Figure 8—table A

Number of radioactive E coli in lung homogenates of ethanol treated guinea pigs various times after exposure

Experiment	Hours after exposure 0	1	2	3	5
I	463—337	328—270	275—206	146—192	148—163
II	395—356	305—234	309—275	175—164	198—171
III	438—425	296—304	210—264	280—353	253—236
IV	338—397	338—349	240—257	203—235	167—159
V	368—297	327—378	272—163	194—256	183—167
VI	427—368	316—369	300—357	349—332	195—236
VII	251—217	275—138	247—291	280—284	154—158
VIII	360—395	428—430	—	268—267	311—339
IX	220—202	230—232	—	294—256	240—213
X	290—306	356—407	266—225	239—175	301—342
XI	361—341	362—381	296—304	307—307	384—419
XII	217—210	279—249	164—195	255—272	150—123
XIII	243—275	425—400	238—241	251—222	162—139
XIV	235—174	220—219	364—299	283—328	238—284
Mean per cent	100	102	86	87	74
S D per cent units	—	24	29	33	29

Figure 8—table B

Number of viable E coli in lung homogenates of ethanol treated guinea pigs various times after exposure

Experiment	Hours after exposure 0	1	2	3	5
I	106—107	129—112	101—96	42—29	22—13
II	191—221	200—233	114—118	48—73	11—12
III	412—430	205—202	66—67	38—35	7—9
IV	253—275	231—207	87—74	18—24	8—4
V	299—356	244—250	53—62	14—28	10—14
VI	301—270	267—261	122—81	25—28	21—27
VII	375—357	336—382	112—140	98—103	16—25
VIII	330—289	218—212	110—125	33—38	9—8
IX	264—243	307—328	100—109	38—36	16—15
X	286—303	269—240	140—127	48—56	13—17
XI	433—370	291—279	200—182	91—77	26—20
XII	425—419	422—435	62—68	43—45	21—19
XIII	448—471	403—401	245—258	75—87	22—22
XIV	525—578	517—519	137—155	48—39	49—42
XV	473—443	550—507	244—251	43—47	24—25
Mean per cent	100	91	40	15	6
S D per cent units	—	20	20	9	4

Figure 9—table A

Number of radioactive E coli in lung homogenates of coal dust exposed guinea pigs various times after exposure

Experiment	Hours after exposure 0	1	3
I	299—325	256—243	239—248
II	285—277	204—185	126—143
III	285—297	217—202	170—161
IV	474—414	263—272	377—330
V	426—432	262—280	179—185
VI	362—314	351—336	298—305
VII	314—322	252—261	326—362
VIII	315—301	229—238	304—279
IX	423—371	315—315	350—336
X	225—208	247—221	222—205
XI	198—213	173—206	153—164
XII	299—277	281—301	212—175
XIII	170—201	145—138	92—84
XIV	232—200	241—238	194—181
XV	226—248	185—173	134—112
Mean per cent	100	83	74
S D per cent units	—	16	21

Figure 9—table B

Number of viable E coli in lung homogenates of coal dust exposed guinea pigs various times after exposure

Experiment	Hours after exposure 0	1	3
I	262—268	191—161	90—87
II	223—244	138—126	69—52
III	298—270	184—195	118—106
IV	177—175	281—294	78—109
V	105—120	78—87	82—82
VI	143—146	110—122	36—45
VII	71—85	104—101	20—26
VIII	224—210	154—167	100—126
IX	157—171	146—158	88—94
X	222—227	160—187	116—150
XI	110—89	177—158	69—70
XII	105—103	78—89	26—34
XIII	111—122	99—122	53—41
XIV	118—155	115—107	23—25
XV	154—148	65—84	73—63
Mean per cent	100	90	43
S D per cent units	—	36	17

Figure 9—table C

Number of radioactive E coli in lung homogenates of chronic exposure control guinea pigs various times after exposure

Experiment	Hours after exposure 0	3
I	245—244	131—121
II	238—262	138—131
III	231—209	189—200
IV	311—306	212—214
V	235—232	219—196
VI	216—239	212—235
VII	265—269	229—209
VIII	215—341	191—165
IX	251—232	184—206
X	207—214	108—129
XI	231—216	152—140
XII	297—313	207—195
XIII	228—225	187—208
XIV	185—206	176—181
XV	354—354	246—254
Mean per cent	100	74
S D per cent units	—	15

Figure 9—table D

Number of viable E coli in lung homogenates of chronic exposure control guinea pigs various times after exposure

Experiment	Hours after exposure 0	1	3
I	211—218	149—151	48—49
II	191—186	121—151	29—29
III	157—194	171—145	44—40
IV	154—205	110—121	72—65
V	149—125	—	44—24
VI	139—147	—	34—30
VII	33—42	—	19—8
VIII	94—99	—	18—25
IX	133—145	—	12—11
X	120—114	—	46—52
XI	152—125	—	13—13
XII	538—542	335—333	83—92
XIII	295—271	—	60—64
XIV	377—367	197—218	86—94
XV	585—661	494—484	53—40
Mean per cent	100	72	22
S D per cent units	—	14	11

Figure 10—table A

Number of radioactive E coli in lung homogenates of cigarette smoke exposed guinea pigs various times after exposure

Experiment	Hours after exposure 0	3
I	225—227	180—122
II	339—381	209—199
III	202—226	374—370
IV	326—284	315—327
V	212—217	213—289
VI	270—275	199—178
VII	236—234	195—209
VIII	260—253	168—170
IX	349—232	314—372
X	238—269	202—186
XI	163—147	245—275
XII	225—283	175—169
Mean per cent	100	98
S D per cent units	—	40

Figure 10—table B

Number of viable E coli in lung homogenates of cigarette smoke exposed guinea pigs various times after exposure

Experiment	Hours after exposure 0	3
I	138—121	18—22
II	229—207	60—61
III	102—97	80—77
IV	188—163	28—47
V	135—118	23—37
VI	109—104	11—12
VII	65—68	38—35
VIII	25—34	9—14
IX	311—305	48—45
X	156—155	47—39
XI	192—208	24—30
XII	339—375	107—96
Mean per cent	100	30
S D per cent units	—	20

Table 8 — addendum

Calculation of mechanical elimination of viable E. coli expressed as percent of reduction of viable bacteria (m) for various periods after exposure. The different dilutions of T and V_L have been corrected for in the bottom calculation.

Symbol	Reference	Period after exposure, hours: 0—½		½—1		1—2	
f	table 5	4.8		3.6		7.7	
T	table 6	164	113	113	67	67	34
V_L	table 6	145	131	131	106	106	55
$\bar{v}_T$	$\frac{T(t_1)+T(t_2)}{2}$	139		90		51	
V_T	$f \times \bar{v}_T$	667		324		393	
$V_L(t_1)-V_L(t_2)$		14		25		51	
m	$\frac{V_T \times 100}{V_L(t_1)-V_L(t_2)}$	48		13		8	

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BY

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FROM THE DEPARTMENTS OF PHYSIOLOGY AND PHARMACOLOGY

UNIVERSITY OF GÖTEBORG, SWEDEN

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INTRODUCTION

It seems obvious that any estimation of the exact amount of noradrenaline (NA), released at the adrenergic nerve terminals requires procedures for a total blockade of the local mechanisms for transmitter elimination in order to allow a quantitative escape to the blood stream However since the nature of these elimination mechanisms was largely unknown until fairly recently only a few studies concerning the NA amounts released upon graded sympathetic stimulations are available and they are mainly confined to the spleen Thus attempts have been made with bioassay methods to estimate the NA release per impulse from this organ, which in several respects is highly specialized with a large bulk of richly innervated smooth muscles of both vascular and capsular origin (Brown and Gillespie 1957, Haefely Hurlimann and Thoenen 1965) It is not excluded however that the spleen might pose some difficulties in this respect since it has been claimed that so far unidentified pressor agents *per se* unrelated to the transmitter, are released into the blood stream upon splenic nerve stimulation (cf Allela *et al* 1960)

Whichever the case there is virtually no quantitative information about the NA release from other types of well defined adrenergic neuroeffectors and almost nothing appears to be known about the details of the NA release at the level of the individual varicosity It was therefore considered to be of interest to perform such a study particularly since considerable structural and histochemical information has recently been gathered about the adrenergic neuron its axonal ramifications fibre varicosities and their effector contacts their contents of adrenergic granules and NA (cf Acta physiol scand 1966 *67* 3–4) The vascular bed of the cat calf muscles was chosen as a suitable target tissue

Since as mentioned a blockade of the local elimination mechanisms for the adrenergic transmitter is a prerequisite for such a study it was combined with an analysis of the normal routes of NA elimination in the target tissue chosen

In principle the adrenergic transmitter may be eliminated by three different mechanisms 1 by diffusion to the blood stream 2 by local enzymatic destruction 3 by reabsorption into the nerve fibres Concerning route 1 it was earlier generally assumed that the adrenergic transmitter in contrast to the cholinergic one was indeed eliminated mainly by diffusion to the blood stream (cf Rosenblueth [illegible] However observations concerning the behaviour of adrenergically innervated vascular smooth muscles suggested that some type of specific local [illegible] transmitter elimination must be involved (Folkow 19[illegible]) though its [illegible] remained unknown Whatever the nature of this [illegible]

be able to cope most efficiently with the release of the adrenergic transmitter over the entire range of physiological discharge frequencies

This does not deny that generally a fraction of the released adrenergic transmitter seems to be eliminated by diffusion to the tissue spaces and the blood stream (over flow) For example Euler and Luft (1951) calculated that 0 5–1 0 μg of NA per minute escapes into the circulation in normal man However this overflow fraction seems in most situations to be too small to significantly affect the behaviour of adrenergically controlled effectors It increases markedly if the adrenergic fibres are stimulated at supraphysiological rates and may then produce some remote effects (Celander 1954) In such situations the local elimination mechanism(s) can apparently no longer keep pace with the rate of NA release This leads among other things to a local transmitter accumulation as revealed by an often marked retardation of the normally prompt poststimulatory relaxation of the vascular smooth muscles (Folkow 1952)

Further the overflow fraction also increases considerably in skeletal muscles if exercise hyperemia is superimposed on a steady vasoconstrictor fibre activity (Carlsson Folkow and Haggendal 1964) These findings are in agreement with earlier observations that the NA blood levels in man increase significantly during muscular work (e g Vendsalu 1960)

The question then arises which of the two principles for *local* elimination that are primarily responsible for the normal removal of the adrenergic transmitter Concerning route 2 the local enzymatic destruction monoamine oxidase (MAO) appears to exert its function within the adrenergic neurons rather than at the neuroeffector junctions (cf Carlsson 1965) Catechol O methyltransferase (COMT) is mainly responsible for the efficient hepatic inactivation of blood borne catechol amines (Lund 1951 Axelrod and Tomchick 1958 Carlsson and Waldeck 1963) but appears to be of only minor importance for the local transmitter elimination (Axelrod 1966 Andén Henning and Magnusson 1967 unpublished results see also Iversen 1967) Further the fact that no net change in the outflow of NA metabolites could be observed following sympathetic stimulation of the skeletal muscles in dogs which had received labelled NA indicates that local enzymatic destruction is on the whole of little relevance for the elimination of the adrenergic transmitter (Rosell Kopin and Axelrod 1963) Enzymatic destruction may however be relatively more important for exogenous noradrenaline

Concerning route 3 on the other hand the active NA reuptake by means of a membrane pump there is now considerable evidence to suggest that this may be by far the most important mechanism for the local elimination of the adrenergic transmitter (for reviews see Carlsson 1965 Iversen 1967 and Hamberger 1967) It is possible that still other so far unknown inactivating mechanisms are involved in the local transmitter elimination but if so they must be of subordinate importance compared with those discussed above

In the present study specific drugs were used to block the enzymatic break down

of NA as well as the active reuptake mechanism which otherwise allows the tran
mitter to re enter the fibre terminals Concerning the NA release from the isolate
calf muscle preparation before during and after graded sympathetic stimulation
chemical methods for NA detection in the arterial blood and in the venous effluer
were employed Further certain characteristics of the smooth muscle responses
the blood vessels and the nictitating membrane which often can reveal disturbanc
in the transmitter release and elimination were also studied Advantage was he
taken of earlier observations concerning the frequency response relationships an
the fact that any delay in the local transmitter elimination tends to greatly prolor
the poststimulatory relaxation of e g vascular smooth muscle (Folkow 1952
—Part of the present results has been briefly outlined in an earlier preliminar
report (Folkow and Haggendal 1967)

METHODS

I Experiments for direct measurements of the NA release

Experiments were performed on 22 cats whose bodyweights were between 3 and 5 kg They were anesthetized and prepared as in an earlier study (see Carlsson, Folkow and Häggendal 1964) using the same technique and equipment with the exception that in the present study the muscles were perfused in the normal way ("constant pressure perfusion") instead of using "constant flow perfusion" by means of a pump

The intact calf muscles of the left hind limb were carefully isolated from the rest of the animal with the exception of the femoral artery and vein and the sciatic nerve The skin and paw circulation was excluded from the calf region The right adrenal gland was extirpated and the left one completely denervated The small and large intestines were also extirpated The left abdominal sympathetic trunk was centrally cut cautiously isolated and put in a ring electrode It was stimulated with square wave stimuli at the level of L_4–L_5 usually at a frequency of 6 imp/sec a pulse duration of 3 msec and a voltage of 5–7 In each experiment it was carefully checked that the voltage was kept well above the level that induced the maximal effector response for the given frequency thus assuring that all fibres were activated throughout Muscular work was induced by stimulation of the intact sciatic nerve with 2 or 4 imp/sec In this case voltage was kept around 0 5–1 with a pulse duration of 0 2 msec which is far below the strength needed to activate the postganglionic vasomotor fibres within the sciatic nerve

After heparinization the arterial blood pressure was recorded by a mercury manometer and the venous effluent from the calf muscles was monitored by an optical drop recorder which operated an ordinate writer By a side tube the total venous effluent from the calf region could be collected for catecholamine analysis arterial blood samples were usually taken simultaneously Each sample generally amounted to 13–15 ml and the catecholamine content was estimated according to Häggendal (1963)

To compensate for the blood loss imposed by taking blood samples two different techniques were used Either 25–30 per cent of the blood volume of the animal was withdrawn at the onset of the experiment with compensation in the form of an equal amount of dextran Tyrode solution The blood thus collected was then diluted to a corresponding degree as the circulating blood volume and when blood samples were taken during the experiment equal amounts of the diluted blood were infused However in most experiments blood taken from another cat was

given as a substitute Since big donors were used 250–300 ml of blood moderately diluted by dextran could usually be collected In order to remove catecholamines from this blood it was slowly infused via the portal vein of the recipient cat for catecholamine clearance in the liver before the experimental procedures were started An equal amount of blood was then drained from the recipient via its femoral vein In four of the experiments these massive transfusions of homologous blood caused obvious rheological disturbances because of partially incompatible blood These experiments were discarded since the blood content of catecholamines then generally rose to high levels

A full procedure for taking blood samples was organized as follows After arterial and venous blood samples had been taken during a period of rest the sympathetic stimulation was initiated and new blood samples were collected after a 6 min period of continuous stimulation to allow for steady state conditions After 2 pairs of samples had been taken the sympathetic stimulation was stopped Muscular work was now started and blood samples were taken after 15 min of steady exercise The sympathetic stimulation was then started again while muscle exercise was still going on and after 6 min of continuous sympathetic stimulation blood was again collected if possible as two series of samples The combination of exercise and sympathetic stimulation was performed because it had earlier been observed that this procedure increased the NA escape (Carlsson Folkow and Haggendal 1964) furthermore when muscle blood flow is low a delay in the escape of labelled NA has been reported (Rosell Kopin and Axelrod 1963) Fifteen minutes after cessation of the sympathetic stimulation and/or after 15 minutes of muscle rest control arterial and/or venous blood samples were taken Thereafter the drugs for blockade of NA reuptake and/or enzymatic breakdown were given *i v* (some times *i a*) and about 15 minutes later the entire procedure for sample collection or its most relevant parts was repeated Since the blood flow was continuously measured it could be checked whether any failure in the vaso constrictor fibre action occurred during the period of sample collection Care was taken to maintain blood pressure constant and the animal in a good general condition

It was however often not possible to carry out the entire procedure for sample collection or to obtain frequent values for the catecholamine content in arterial blood because the total amount of blood used for compensation of the blood loss was limited Then the experiment was concentrated on some aspects of the NA release only e g the effect of membrane pump blockade Sometimes the values for the venous blood at rest had to be used for the calculation of the amount of NA released per unit time but this seemed to be justified since it was earlier observed that the resting AV difference for catecholamine content was usually quite small (see also below) with low values for both arterial and venous blood contents of NA

For blockade of the active reuptake of NA into the nerve terminals the membrane pump blockers desipramine (DMI) 1 to 3 mg/kg *i v* or 1 phenyl 1 (3 methyl aminopropyl)3 3 dimethylphthalane (Lundbeck 3 010) 2 to 10 mg/kg *i v* or 0 1

to 10 mg i.a. were used. In some of the cats attempts were made to simultaneously block the membrane pump as well as COMT and/or MAO by giving DMI or Lu 3 010 and, in addition, α-n-propyl dopacetamide (H 22/54, Hässle) 100 mg/kg, dopacetamide (H 13/49, Hässle) 0.8 g/kg or tropolone acetamide (H 17/27, Hässle) 20 mg/kg and nialamide 100 mg/kg, respectively.[1]

II. The effect of membrane pump and enzyme blockers on the responses of adrenergically innervated smooth muscle

These experiments were performed on 29 cats, anesthetized as earlier described.

1. In 19 of the animals the nictitating membrane (n.m.) was studied; in 10 of these cases the right n.m. had been denervated postganglionically 5–7 days before hand, to be used as a virtually nerve free smooth muscle control to the left n.m. in the subsequent acute study. Both n.m. were then connected to either isotonic or isometric levers and their responses to intravenously or close intra-arterially injected drugs and to graded stimulations of the centrally cut postganglionic fibres (to the left n.m.) were studied. Great care was taken to adjust voltage and pulse duration to such levels that *all* postganglionic fibres were stimulated throughout. Different rates of stimulations (0.5/sec up to 32/sec) were employed in order to study the frequency response relationship and the rate of relaxation of the smooth muscles before and after administration of the membrane pump blockers and/or the enzyme blockers.

2. In 10 cats the effects of graded vasoconstrictor fibre stimulations were studied on the same type of calf muscle preparation as described above under I. The cholinergic vasodilator fibres were blocked with atropine (0.3 mg/kg) and the voltage and pulse duration were so chosen as to activate *all* the vasoconstrictor fibres to the calf muscles at the different rates of stimulation employed. The vasoconstrictor responses and, especially, the rate of vascular relaxation upon cessation of the vasoconstrictor fibre stimulation were recorded before and after administration of the membrane pump and/or enzyme blockers.

[1]) For generous supply of the mentioned drugs we want to thank AB H. Lundbeck, Malmö and AB Hässle, Mölndal, Sweden.

RESULTS

I Extent of transmitter release

1 *Resting conditions*

When no sympathetic activity was going on the mean arterial NA concentration was 1 4±0 06 (S E M) ng/ml plasma ($n=9$) while the mean venous NA concentration was 1 2±0 04 ng/ml plasma ($n=9$) There was thus if anything a tendency of NA uptake but the AV difference was not statistically significant

When however membrane pump blockers had been given the corresponding figures were moderately increased especially for the venous NA content being 2 2±0 14 ($n=9$) and 2 7±0 16 ($n=9$) respectively Therefore there was now evidence of a slight spontaneous release of NA ($p<0\ 05$ in paired analyses) Further the figures for the oppositely directed AV differences before and after the membrane blockers differed significantly ($p<0\ 02$)

2 *Vasoconstrictor fibre activity*

a During muscle rest When the vasoconstrictor fibres were stimulated at 6 imp/sec while the skeletal muscles were at rest a regional reduction of blood flow could freely occur The NA escape from the calf preparation to the blood stream amounted in this situation to 9±2 (S E M) ng/ml for 100 g of muscle as calculated from the arteriovenous NA difference and the blood flow This and the subsequent mean values also summarized in Fig 1 are taken from five experiments where it was possible to carry out the full procedure for sample collection (see Methods) under technically satisfactory conditions This illustration is mainly intended to indicate the relative changes in NA release that are induced by exercise and/or membrane pump blockers In some other experiments the NA escape was lower than the given figure

The *total* NA amount appearing per minute in the venous effluent per 100 g of resting calf muscles would during such a sympathetic stimulation be 9+1 4×2 i e about 12 ng/min as an average The figure 9 represents the abovementioned NA escape/min and 1 4 the arterial plasma concentration of NA at rest The figure 2 represents the average plasma flow in ml/min per 100 g of resting muscle at this rate of sympathetic stimulation Before the sympathetic stimulation the venous effluent would as an average contain 1 2×6 i e about 7 ng/min where 1 2 represents the venous plasma concentration of NA at rest and 6 the average plasma flow in ml/min per 100 g of resting muscle at normal basal vascular tone In other w

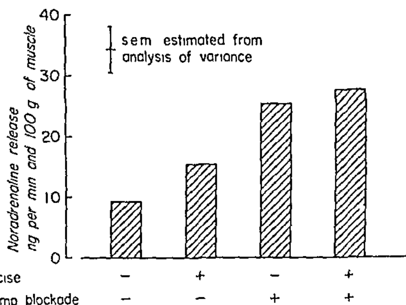


Fig. 1. Schematic illustration of the average NA escape from the calf muscles of 5 cats, calculated as ng/min per 100 g of tissue at a constant rate of sympathetic stimulation at 6 imp/sec. In these 5 experiments the full procedure for sample collection (see Methods) could be carried out in a technically satisfactory way with no evidence of fibre damage. The untreated group differed significantly from the group treated with membrane pump blockers ($p < 0.025$) and from the one treated with membrane pump blockers plus muscle exercise ($p < 0.01$). The bar indicates the standard error of the means. Statistics were calculated according to analysis of variance.

these admittedly approximate deductions suggest that the NA amount delivered to the general circulation from the vascular bed of resting muscle generally increases only slightly when the blood flow is reduced by the vasoconstrictor fibres. Even if these fibres were activated at rates within the higher physiological range, i.e. 6 impulses per second, only some 5 ng of NA/min would then be added per 100 g of skeletal muscle. This is in general agreement with earlier results (Carlsson, Folkow and Häggendal 1964).

b. During exercise. As was also observed in these earlier experiments, initiation of exercise, with the creation of a stable, nearly maximal exercise hyperemia, leads sometimes to a considerable increase of the NA release for the same extent of vasoconstrictor fibre activity. Compared with the situation during muscle rest, the average NA escape from the calf preparation to the blood stream increased about twofold in the five experiments shown in Fig. 1, to 16±3 ng/min per 100 g of muscle. In other experiments, where only part of the full procedure for sample collection could be carried out in a satisfactory way, the NA escape during exercise and sympathetic stimulation at 6 imp/sec could reach values around 30 ng/min. Thus the combination of sympathetic stimulation and exercise always implied an increased NA escape but the extent of this increased escape could vary considerably

c During muscle rest after membrane pump blockade Administration of membrane pump blockers (DMI 3 mg/kg i v or Lu 3 010 usually 2 mg/kg i v) increased the average NA escape from the resting calf muscles to the blood stream to 26±5 ng/min per 100 g of muscle when the sympathetic fibres were stimulated at 6 imp/sec (Fig 1) The highest figures observed in these five and other experiments under these experimental circumstances were of the order of 35 ng/min per 100 g of muscle

d During exercise after membrane pump blockade For the mentioned rate of sympathetic stimulation the NA escape to the blood stream appeared to increase somewhat further when the administration of a membrane pump blocker was combined with exercise The average NA escape to the blood stream then reached a figure of 28±4 ng/min per 100 g of muscle in the five experiments illustrated in Fig 1 The highest figures for NA release observed in technically satisfactory experiments under these experimental circumstances were about 40 ng/min per 100 g of muscle Several of these figures were obtained in experiments where only part of the full procedure was performed and are therefore not included in the mean values given in Fig 1

In some of the present experiments COMT and/or MAO inhibitors were given in addition to the membrane pump blockers or alone but in no case did this significantly increase the NA release from the calf preparation during sympathetic stimulation On the other hand, administration of the COMT inhibitor gradually increased the NA content in the arterial blood even when given as a close arterial infusion to the limb In all likelihood this reflects a blockade of the efficient liver elimination of NA via the hepatic COMT

The figures mentioned for NA escape after membrane pump blockade and during exercise call for some considerations already in this connection The highest figures given above were all obtained when blood samples were withdrawn within 6–15 min after the start of the continuous sympathetic stimulation In some experiments where samples were taken also after this period of time these samples usually revealed a tendency of decline in the NA release This decline could hardly be ascribed to e g fibre damage because a fairly short period of adrenergic fibre rest was usually enough to restitute a largely normal vasoconstrictor response to repeated sympathetic stimulation It may therefore mainly represent a reversible gradual and minor exhaustion of the transmitter release of the same type as is described below in section II and illustrated in Fig 2 panel B but occurring more slowly since the frequency of stimulation was lower Therefore while the membrane pump blockers seem to induce an apparently complete escape of the adrenergic transmitter to the blood stream they might also because of this large escape cause a small gradual decline in the transmitter quantity released per stimulus at least at the relatively high stimulation frequency used in these experiments

In any case it is possible that a slight decline in NA escape may sometimes have started even earlier thus affecting some of the samples taken during earlier

of the stimulation period These samples could on the other hand hardly be taken much earlier after the onset of the stimulation since sufficient time must be allowed to make certain that the NA escape to the blood stream had reached an equilibrium This tendency of a slowly declining NA release after membrane pump blockade as well as an accidental occurrence of some nerve fibre damage and local trapping of blood seems unavoidable in experiments of this type Such factors probably explain why some of the present measurements gave relatively low values for NA escape and why the spread between the values could sometimes be considerable Consequently the mean values of Fig 1 would tend to *underestimate* the true NA release especially after membrane pump blockade Therefore these mean values mainly serve to illustrate the general trend of changes taking place with the different procedures For such reasons it is likely that the *highest* values obtained for NA release after pump blockade are the most representative ones for the true transmitter release However even these values which as mentioned were about 40 ng/min per 100 g of muscle at a frequency of 6 impulses per second will probably imply a slight underestimation, if anything

It may further be questioned whether the membrane pump blockade was complete Other experiments (Waldeck 1967) however show a virtually complete blockade of the uptake of ^{3}H NA by the doses of blocking agents used in the present experiments To explore whether this was the case also in these experiments increasing amounts of DMI or Lu 3 010 were given intra arterially in a few experiments while a continuous sympathetic stimulation was performed This did not however materially increase the NA escape on the contrary when very high local concentrations of the membrane pump blockers were created (e g intra arterial injection of 0 5–4 mg Lu 3 010 to 100 g of muscle) there was often an abrupt fall in NA escape This suggests a temporary interference with the NA release at the same time the vascular response to the stimulation vanished for a considerable period (see also section II 2 below)

II Smooth muscle responses

1 *Nictitating membrane*

a Before membrane pump blockade Fig 2 illustrates a characteristic n m response where a continuous postganglionic stimulation of supramaximal strength was applied while the frequency was varied in a stepwise fashion When supraphysiological frequencies were employed (cf Folkow 1955) in this particular case 16 and 32 imp/sec the membrane response initially increased somewhat but it gradually returned to slightly submaximal levels despite constant stimulation It was a general phenomenon that a steady contraction level could be maintained only at frequencies below 12–14 imp sec At all higher frequencies on the other hand there was a decline in the response after an initial peak and this decline was more rapid the higher the supraphysiological frequency (see Fig 2) Further intermittent but brief

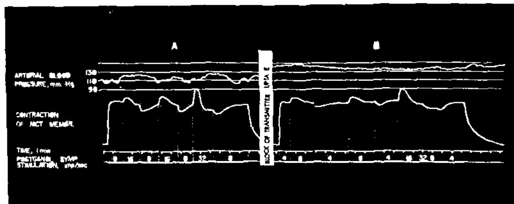


Fig 2 Cat 3 kg Chloralose Supramaximal po tganglionic sympathetic stimulation to the nictitating membrane isotonic recording In panel A a steady level of contraction is maintained at 8 imp/sec which was also the case with 12 imp/sec When the frequency is directly shifted over to 16 or 32 imp/sec there is a rapid increase in contraction level followed by a gradual relaxation in spite of continued stimulation Note that this relaxation is more rapid at the higher of these two supraphysiological frequencies Upon return to 8 imp/sec during continued stimulation there is after a transient relaxation a slow regain to the earlier contraction level

In panel B after blockade of NA reuptake (4 mg/kg Lu 3 010) 4 imp/sec now gives about the same degree of contraction as earlier 8 imp/sec illustrating a displacement of the frequency response curve to the left (ee text) Further a steady level of contraction can now be maintained at a frequency of 4 imp/sec while 8 imp/sec fails to do so A regain of contraction level is however obtained when the frequency is reduced to 4 imp/sec Note the delayed relaxation upon cessation of the stimulation

interruptions of a supraphysiological stimulation usually resulted in a better preservation of the contraction level throughout a prolonged stimulation period These observations are in favour of a failing transmitter release per stimulus at uch high stimulation rates and do not suggest any smooth muscle fatigue especially not since a stepwise reduction from a supraphysiological to a physiological frequency of stimulation generally caused a *regain* of the n m response after a slight transient decrease (see Fig 2) Failure of impulse transmission along the axons seems excluded since C-fibres in general appear to be able to transmit impulses at far higher rates than those employed here

b After membrane pump blockade Administration of a membrane pump blocker (usually Lu 3 010) induced the following effects on the n m in most cases Even though no sympathetic stimulation was applied the *acutely* denervated n m (nerve fibres cut 1–3 hours earlier) usually increased its tension very slowly when the blocker was given It ultimately reached a fairly steady contraction level which in most cases was at most some 20 per cent of that produced under normal circumstances by a stimulation of 1 imp/sec The *chronically* denervated n m on the other hand which was usually at least ten times more sensitive to injected NA,

did not show any corresponding slow contraction when I u 3 010 was given This indicates that the slow contraction of the acutely denervated n m is a matter of a slow *local* release of transmitter from the adrenergic nerve terminals even when not stimulated

However this local release and that occurring in all other intact sympathetic pathways that normally fire at low rates is evidently too small to bring about any considerable increase in blood borne NA despite the blockade of the membrane pump Had this been the case the chronically denervated sensitized n m would have revealed the presence of such NA concentrations by responding with a contraction Moreover after membrane pump blockers the NA levels in the arterial blood were generally only slightly increased according to the biochemical estimations (see section I)

Further the acutely denervated n m responded with somewhat larger and more prolonged contractions to i v or i a injected NA when the membrane pump blocker had been given No such potentiating effect was seen in the chronically denervated n m beyond the level of sensitization that was already present at the onset of the experiment suggesting that the membrane pump blocker does not directly potentiate the effector response to a given amount of NA This enhancement and prolongation of the responses of the innervated n m to injected NA is therefore evidently a consequence of the blockade of the membrane pump This blockade appears to cause a higher and better maintained local NA concentration at the neuroeffector junctions (Trendelenburg 1963)

Postganglionic sympathetic stimulation after blockade of the membrane pump generally resulted in the following changes The frequency response relationship was usually displaced to the left This appeared to be essentially due to the fact that instead of causing as earlier rapidly established stable levels of contraction for a given frequency of stimulation the n m now usually exhibited slowly climbing responses after an initial steep rise especially to low frequency stimulation Further upon interruption of the stimulation the relaxation was clearly prolonged in all cases Both these types of changes are expected consequences of a blockade of the membrane pump mechanism since this blockade ought to lead to a local accumulation of the transmitter No such effects were seen when COMT and MAO inhibitors were given suggesting that these enzymes are not to any significant extent involved in the local elimination of the adrenergic transmitter

Moreover after blockade of the membrane pump prolonged stimulations at higher rates revealed a tendency of response decline already at the higher range of physiological frequencies which before the block could maintain a steady contraction This is illustrated in Fig 2 when section B is compared with section A Before the membrane pump blockade a steady level of contraction was easily maintained at a frequency of 8 imp/sec and this was also the case up to 12–14 imp/sec

There was however after administration of the blocking substance evidence of a reduction of this critical rate of stimulation to below 8 imp/sec It should be

noted that this change occurred despite the tendency of local transmitter accumulation caused by the blockade of the membrane pump which—other factors being constant—would better maintain any given neurogenic response of the n m This will be further discussed below

2 *Blood vessels*

The most characteristic effect of the membrane pump blockade on the vascular responses was the delayed relaxation upon cessation of stimulation Potentiation of responses to brief periods of constrictor fibre stimulation or to i a NA injections was also seen but these effects were not as pronounced and clearcut as the delayed relaxation This prolongation of the normally quite rapid vascular relaxation upon interruption of the stimulation could be very pronounced as is illustrated in the upper and especially the lower sections of Fig 3 The upper section of this figure illustrates the delayed relaxation in more detail while the kymograph speed is increased The lower section of Fig 3 further illustrates the earlier described phenomenon that vascular relaxation becomes considerably delayed even if no pump blockade has been induced if the constrictor fibres for a brief period are stimulated at supraphysiological rates beyond 8–10 imp/sec (compare A and B in the lower section of Fig 3) This phenomenon has been ascribed to an excess of transmitter accumulation in the early phase of the period of supraphysiological stimulation, presumably because the local elimination of the transmitter is then unable to cope with the abnormally high rate of NA release (Folkow 1952) The transmitter may then by diffusion reach the inner layers of vascular smooth muscle which are not innervated (Falck 1962 Fuxe and Sedvall 1965) Since there are no fibre varicosities here and hence no reuptake mechanism for NA the transmitter may be expected to be only slowly eliminated from the receptor sites However as was also the case with the n m prolonged sympathetic stimulation at supraphysiological rates soon results in gradually declining vascular responses This is probably due to the fact that after a short period of high frequency discharge the NA amount released per impulse starts to decline to subnormal levels

However the local accumulation of the transmitter occurring at supraphysiological discharge rates if the stimulation period is not too prolonged no doubt becomes markedly enhanced once the membrane pump is blocked While normally the vessels relax within a fraction of a minute upon interruption of a constrictor fibre discharge (see upper section of Fig 3) the relaxation can last 7–8 minutes after membrane pump blockade (see Fig 3 lower section C and D) In fact the effects of brief stimulation periods then simulate the prolonged responses normally seen when noradrenaline is administered in large doses as an intra arterial injection No such effects were seen when COMT and/or MAO inhibitors had been given

As mentioned the responses to constrictor fibre stimulation or to injected adrenaline were usually potentiated when small or moderate amounts of

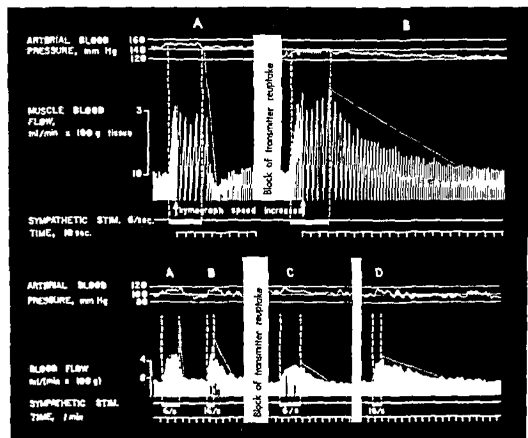


Fig 3 *Upper section* Cat 3 5 kg Chloralose Effect of supramaximal vasoconstrictor fibre stimulation at 6 imp/sec upon calf blood flow Note the prompt vascular relaxation with a transient poststimulatory hyperemia upon sudden cessation of this physiological discharge rate (panel A) while the relaxation is markedly retarded after blockade of NA reuptake by DMI 3 mg/kg (panel B)

Lower section Cat 3 kg Chloralose Same type of preparation but here the vasoconstrictor fibres are stimulated both at 6 imp/sec (A) and at a supraphysiological rate 16 imp/sec (B) Note the obvious delay in vascular relaxation upon the cessation of stimulation at this higher rate suggesting a local accumulation of transmitter After administration of Lu 3 010 10 mg/kg the vascular relaxation after stimulation at both 6 imp/sec (C) and especially at 16 imp/sec (D) is profoundly retarded

pump blockers had been given However if very large amounts of Lu 3 010 had been given e g 10 mg/kg as an intra arterial infusion the drug appeared to exert some α blocking effect since both the responses to NA injection and to nerve stimulation then became more or less temporarily depressed occasionally they were almost totally blocked It is probably because of such an α blocking effect that the neurogenic responses in Fig 3 lower section are somewhat depressed after administration of Lu 3 010 However it has already been mentioned that such high doses of Lu 3 010 might interfere with the NA release as well

DISCUSSION

I Extent of transmitter release

1 *NA release during fibre rest*

When no membrane pump blocker had been given there was if anything evidence of a slight uptake of NA in agreement with several earlier investigations (cf Iversen 1967) After membrane pump blockade on the other hand the present results suggest a minor spontaneous NA release from the calf muscles (see also Rosell Kopin and Axelrod 1963) Further also the behaviour of the acutely denervated m m revealed a slow spontaneous release of endogenous NA The extent of this release was difficult to evaluate, but it appears to correspond to only a small fraction of that released during stimulation at 1 impulse per second as judged from the effector responses It seems likely that this slight spontaneous release of NA reflects the subthreshold random quantal release known to occur for transmitters in general (cf Katz 1962 Burnstock and Holman 1961 1962 a b) Since the membrane pump was blocked these subthreshold NA amounts could accumulate locally until eventually concentrations might be reached which were high enough to produce effector excitation and a weak contraction

2 *NA release during sympathetic stimulation*

a Release per stimulus as related to total NA content The highest figures of NA release for a given rate of sympathetic stimulation were seen during exercise hyperemia when the membrane pump mechanism had also been blocked by DMI or Lu 3 010 Addition of COMT or MAO blockers appeared to have no conspicious influence in this respect The possibility that so far unknown inactivating mechanisms play a role for the local transmitter elimination can of course not be excluded However if this is the case it seems very unlikely that they should be of any substantial importance compared with the routes for transmitter elimination outlined in the Introduction

It seems unlikely that any of the procedures involved in the present experiments should lead to any significant *enhancement* of the transmitter quantity released per stimulus though such a possibility cannot be entirely excluded from a theoretical point of view Most of the experimental procedures appear after all to act in the opposite direction if anything such as accidental fibre damage local trapping NA by poor perfusion of fractions of the muscle preparation incomplete of the membrane pump or gradual decline of the released fraction

extraneuronal binding of the released transmitter appears however from what has been mentioned above under 1 to be of little relevance Further the fact that stepwise additions of membrane pump blockers did not increase the NA escape during a continuous period of stimulation seems to exclude that the pump blockade should have been grossly incomplete Moreover the fact that the substances used here produce a virtually complete blockade of ³H NA uptake (Waldeck 1967) makes it unlikely in the present experiments that any substantial NA reuptake should have remained

Since most circumstances would if anything tend to decrease the NA output it is likely that the *highest* figures for NA release measured (about 40 ng/min per 100 g of muscle tissue for a stimulation rate of 6 imp/sec) come close to the true total release of adrenergic transmitter at the discharge rate employed though even this figure may slightly underestimate the true release A figure of some 50 ng/min which includes corrections for NA losses during the estimation procedure therefore appears more reasonable for the release per minute from 100 g of skeletal muscle at a discharge rate of 6 impulses per second when all fibres are activated A slightly higher value was given in the preliminary report (Folkow and Häggendal 1967) but a re appraisal of an extended material shows that the above mentioned figure is more valid

Such a figure can be utilized for a very approximate estimation of the fraction of the total NA content that is released per stimulus also the amount released per varicosity may be deduced Admittedly such figures are bound to be affected with considerable errors but they nevertheless serve the purpose to give some idea about the order of magnitude of normal transmitter release Per stimulus the mentioned release corresponds to about 0 15 ng of NA per 100 g of calf muscle

In a recent paper Zimmerman and Whitmore (1967) present figures for the NA release from dog skeletal muscle before and after phenoxybenzamine Unfortunately they do not correlate their figures to either muscle weight or to total muscle content of NA However from their Fig 5 it can be deduced that the NA output per stimulus after correction for losses during the determination process would be close to 0 05 ng after phenoxybenzamine blockade of the NA reuptake Further in another section of the paper it is mentioned that the weight of three muscle preparations averaged 36 g If this weight figure is also valid for the experiments shown in their Fig 5 one arrives at a NA release per stimulus and 100 g of muscle of nearly 0 15 ng This is virtually the same figure as that reported here and in the earlier preliminary report (Folkow and Häggendal 1967) In the study of Zimmerman and Whitmore the stimulation periods were brief so it is very unlikely that any exhaustion of the transmitter release could have interfered

In the present experiments the total calf muscle content of NA was estimated at approximately 8000 ng per 100 g of tissue (see also Sedvall 1964) It then follows that a fraction around 2×10^{-5} of the total store is released per stimulus In case the total NA content of dog muscle is of the same order as in cats Zimmerman

and Whitmore s (1967) results would imply a fraction of essentially the same magnitude It is further interesting that these figures are of the same order of magnitude as the release per stimulus from the adrenal medulla which can be calculated to be about $3\ 5\times10^{-5}$ from Celander s data (1954) In the preliminary report (Folkow and Haggendal 1967) a slightly higher value was given but the present figure seems to be more correct since it is based on a larger material concerning the catechol amine content in the cat adrenal gland Thus Celander s mean value for release per stimulus from one adrenal gland of the cat, as corrected for a body weight of 3 kg was 12 ng The mean *total* content of catecholamines/g cat adrenal gland is 1 1–1 2 mg (see Euler 1956) For a 3 kg cat the weight of the adrenal gland is of the order of 0 3 g This gives $\frac{12}{0\ 3\times1\ 100\ 000}$

Therefore a very good agreement apparently exists between these three figures for NA release per stimulus On the other hand if the relationship between release per stimulus and total NA content is deduced for the spleen (Haefely Hurliman and Thoenen 1965) one arrives at a figure some ten times larger It seems very unlikely that this large difference between the mentioned figures should be only a matter of errors in determination It is then more likely that the fraction of NA release per stimulus can indeed vary between the tissues and that the spleen here for some reason has an especially high turnover of the adrenergic transmitter It will be seen below that a difference of similar magnitude appears to exist concerning the acetylcholine release per stimulus and endplate in the tibial anticus and diaphragma muscles

b Release at the varicosity level Almost all NA in skeletal muscle is present in the varicosities of the vasoconstrictor fibres (Fuxe and Sedvall 1965) and seems to be contained in the adrenergic granules (=vesicles) The number of granules in one varicosity has been calculated to be in the order of about 1500 (Dahlstrom Haggendal and Hokfelt 1966) A figure of about 1000 granules may be more likely as an average and has here been used for the calculations The NA content per varicosity is about 5×10^{-15} g in the rat (Dahlstrom Haggendal and Hokfelt 1966) being in all likelihood of the same magnitude in the cat (Dahlstrom and Haggendal 1966 a) Since the weight of the NA molecule is $2\ 8\times10^{-22}$ g each granule would as an average contain about 15 000 NA molecules In case the fraction of NA release per impulse 2×10^{-5} is valid also at the varicosity level the NA release per impulse from one varicosity would amount to some 400 molecules corresponding to only about 3 per cent of the total content of one NA granule The reported findings of larger vesicles in the varicosities besides those having a diameter of about 500 A will not be discussed in the present paper If they are of functional importance the fraction of NA release will in these granules be even smaller

It will now be discussed whether on the whole a fractional release from the individual granule is likely or whether the transmitter release at this micro level occurs in some other form

It is mainly on the basis of the excellent studies by Katz and coworkers (cf Katz 1962) assumed that transmitters in general are released at random in the form of quantal packets Thus at motor end plates subthreshold 'miniature end plate potentials (min EPP) are produced by this random release It has further been suggested that the quantal packet should correspond to the total content of one acetylcholine (ACh) vesicle partly since they are remarkably constant in size (cf Eccles 1964) The random release of ACh packets is vastly increased upon arrival of an impulse so that 100–300 packets are then almost simultaneously discharged at the end plate producing an EPP (cf Katz 1962 Eccles 1964) Subthreshold miniature potentials occurring at random have also been observed at the adrenergic neuroeffector junctions (Burnstock and Holman 1961)

Several questions arise for example How large is the quantal packet for the adrenergic transmitter how large is the release from each varicosity per stimulus and how many packets may be needed for activation of the average adrenergic effector cell? It is *a priori* possible that one NA packet corresponds either to (1) the entire NA content of one granule or to (2) a small fraction of this content

(1) The alternative that the NA packet should correspond to the *total* content of one adrenergic granule would be a close parallel to the proposed situation at the cholinergic motor end plate However if such an arrangement is on the whole also valid for the adrenergic transmission it is very unlikely that when an impulse arrives the NA granules should be expelled into the junction gap to disintegrate here The adrenergic granules are complex structures for NA synthesis uptake and specific storage Further they seem to be formed in the nerve cell bodies transported down the axons to the nerve terminals where their life span is several weeks (Dahlstrom 1966 Dahlstrom and Haggendal 1966 b) This makes it very unlikely that the NA granules should be lost and destroyed in direct connection with the transmitter release process

This does not on the other hand exclude the possibility that granules expell their *total* NA content into the junction gap during the release process followed by a complete recharging of their stores by means of both active NA reuptake and synthesis At the same time it must however be assumed that the subthreshold miniature potentials occurring at random at the adrenergic neuroeffector junctions (Burnstock and Holman 1961 1962 a b) are derived from NA quantal packets If then one NA granule were identical with such a packet a miniature potential would be derived from the release of as much as 15 000 NA molecules being the approximate granule content Further since Burnstock and Holman s studies suggest that NA must be simultaneously released in the form of several packets to excite an effector cell a joint release of at least one granule from several varicosities—or several granules from one varicosity—in direct contact with the effector would be needed for its excitation

However such a theory can be valid only if it is also compatible with the fact that the fraction of NA released per stimulus can be as low as 2×10^{-5} of the total

transmitter stores as observed in the present experiments Since there seer be about 1000 granules per varicosity the fraction 2×10^{-5} corresponds, as tioned to only approximately 3 per cent of the total NA content of one gra The NA packet can then correspond to one granule only if it is assumed that ac tion of the average adrenergic neuron leads to release of a granule at not than 3 per cent of its varicosities Further even if only e g five NA packet stimulus were needed for threshold excitation of an effector cell, this cell then be in contact with some 150 varicosities to have a chance to become vated at each impulse In fact even in the richly innervated vas deferens about 10–30 varicosities seem to be in contact with the average muscle cell deferens has been calculated to contain 52×10^6 varicosities per cm length (strom Haggendal and Hokfelt 1966) and the same section contains about 4 5 muscle cells (Dahlstrom 1967 personal communication) Occasionally the varicosity may be in contact with up to three muscle cells (Hokfeldt 1967 sonal communication)

It can then be argued that the average effector cell might be excited at only tenth impulse or so because the chances to reach the assumed threshold ar of 5 NA granules would not occur more often if only say 15 varicosities are n tact with each effector cell This would indeed imply a very inefficient ty transmission despite the fact that each quantal packet would create an enoi NA concentration in the junction gap This concentration will amount to 20–40 μg/ml if the contact area varicosity vascular smooth muscle is 1–2 μ the junction gap up to 1000 A wide These considerations alone make it un that the NA packet should correspond to the total content of one granule

Further this hypothesis seems to be untenable also for other reasons Thu characteristics of the junction potentials evoked by graded sympathetic stimu (Burnstock and Holman 1962 a b) are hardly compatible with the view that a tion of adrenergic fibres should only infrequently be accompanied by NA r from their varicosities The results of Burnstock and Holman in addition s that these potentials are constituted by several packets and moreover that a all effector cells are directly affected by the NA release at each stimulus I with respect to well innervated effectors like the pupillary muscle the nicti membrane most blood vessels etc there i little doubt that essentially a effector cells must be activated even at low frequencies of stimulation The ef response of e g the nictitating membrane or pupillary muscle which show rapid single twitches , may reach as much as 50–70 per cent of the maximal res already at 1–2 impulses per second implying that the frequency response cu usually markedly hyperbolic (cf Celander 1954 Folkow 1955) Had the av effector become activated at only e g each tenth stimulus one would have exp the responses to be very weak at such low frequencies (The reason would b the effective frequency for effector excitation once each tenth or fifth se respectively would then be far too low to fuse the single twitches into a tetanic r

se This would be possible first at rates around 10 impulses per second when excitation would occur each second with chances for tetanic summation However such a type of neuroeffector function would result in a very flattened frequency response curve and seems to be incompatible with the normally obtained hyperbolic curves relating stimulation frequency to effector response)

Thus it seems impossible to harmonize both the present figure for the fractional NA release per stimulus and known characteristics of the effector responses with the hypothesis that the *total* NA content of a granule should be identical with the quantal packet

(2) The considerations above make it far more likely that each activation of an adrenergic neuron normally causes NA release from essentially all its varicosities Then the fraction given above 2×10^{-5} would as a rough average hold also for the individual varicosity but this fraction corresponds, as mentioned to only some 3 per cent of the NA content in a single granule which corresponds to a release of some 400 NA molecules per impulse from each varicosity These 400 molecules would thus correspond to the *largest* possible size for the NA quantal packet on the assumption that only *one* packet were released per impulse and varicosity It seems however more likely that these 400 molecules released when a stimulus arrives represent a fusion of still smaller amounts expelled from one or several granules each amount then representing the ultimate packet It would then mean that the random release producing a miniature potential would be equivalent to only a fraction of these 400 molecules According to e g Burnstock and Holman (1962 a b) these miniature potentials vary somewhat in size suggesting that the ultimate packets of NA are not always uniform with respect to number of molecules

Whichever the case with the approximate figures given above for the size of the junction gap 1–2 μ^2 with a width of 1000 A 400 molecules released from a varicosity when an impulse arrives would here create a peak concentration as high as about 0 5–1 μg/ml being about one hundred times above the catecholamine concentration reached in the arterial blood even at intense sympathetic excitation This seems to be a more reasonable NA concentration at the receptor sites than the figure arrived at if the full content of a granule were equivalent with the NA packet

However since the NA quantal packet seems to correspond to only a minor fraction of the content of a granule there must be some mechanism that allows for a fractional release of this content This might be arranged in two principally different ways It is thus possible that each granule contains at least two different pools of NA where a minor fraction is less firmly bound and hence more readily available for release There is indeed considerable indirect support of such a view (cf Carlsson 1965 Iversen 1967) It is further possible that the varicosity membrane with which the granules by their random thermodynamic movements now and then make contact contains areas which have a limited number of release points or attachment sites (cf Katz 1962) for NA to be fired out into the junction gap

If so the quantal release of NA from a granule would be determined *not* primarily by the NA content of the granule but by the number of such membrane receptive sites It is possible that both these principles are utilized in the release of NA so that a small NA fraction of the granules becomes attached to the membrane from where it is 'fired into the junction gap

c Comparison with the cholinergic transmitter release It would seem near at hand to assume that the principles for transmitter release should be largely the same for all systems and species For such reasons the suggestion that the NA packet should correspond to only a minor fraction of the content of one granule might appear less attractive since it seems to be widely assumed that the ACh quantal packet should be equivalent to the full content of a vesicle This may of course not be any valid argument after all since there are some well established differences between the cholinergic and adrenergic transmitter mechanisms for example ACh is en zymatically destroyed locally while NA is in part actively reuptaken in part it diffuses to the blood stream

In any case the present results may justify an exploration of whether the ACh quantal packet might not after all correspond better to only a *fraction* of the ACh content of one vesicle So far there seems to be no exact figures for the number of ACh molecules per quantal packet though the general order of magnitude seems to be clear 10^3–10^4 (Katz 1967 personal communication) Neither is the question concerning the average ACh content of one vesicle definitely settled Estimations of the ACh release per stimulus and end plate have on the other hand been per formed in some mammalian muscles Thus Emmelin and MacIntosh s data (1956) suggest a release of 5×10^{-17} g per stimulus and end plate for cat tibialis anticus muscle If this release is constituted by 200–300 ACh packets as seems to be the case (see MacIntosh 1959 Eccles 1964), each packet would correspond to only some 600–1000 ACh molecules The technique utilized by Emmelin and MacIntosh was so arranged that it is unlikely that a major fraction of the true ACh release could have escaped detection Further the stimulation frequency used 20–25 imp/sec must certainly be considered to be within the physiological range for such a muscle It could therefore hardly have led to any abnormal exhaustion of the transmitter release even if it may be that the release is usually largest during the first few stimuli (cf Eccles 1964)

In case a vesicle contains ACh in isotonic solution some 6000 molecules would be present (cf Eccles 1964) On the other hand according to Emmelin and MacIntosh s data a vesicle would contain only 600–1000 ACh molecules provided that it is identical with the quantal packet The question arises however, whether ACh like NA is instead specifically bound within the vesicle (granule) If so ACh might like NA be present in much higher concentrations since a NA granule appears to contain about 15 000 molecules It may be observed that the majority of the NA granules and ACh vesicles are of about the same size i e 500 A in diameter and that the molecular weight of the two transmitters is about equal Suppose that an

ACh vesicle contains about the same number of molecules as the NA granule then the quantal packet of ACh, as deduced from Emmelin and MacIntosh's figures would correspond to only some 3–5 per cent of the vesicle content This figure is indeed very similar to that deduced from the present results with respect to the NA release per *stimulus* from one adrenergic varicosity

Using the isolated rat diaphragm Straughan (1960) Krnjević and Mitchell (1961) and Mitchell and Silver (1963) have on the other hand given figures for ACh release that seem to be some 10 times higher than those given above for the cat tibial anticus muscle (see also Eccles 1964) The same type of deduction based on the same number of ACh packets per EPP would then give a packet size of about 6–10 000 molecules This figure is no doubt close to the proposed magnitude for the total ACh content of a vesicle

However it is still unsettled whether there may be species or muscle differences both with respect to the number of ACh packets released per stimulus and to the vesicle content of ACh It should be stressed that the diaphragm in many respects is a special muscle differing from most others e g by its higher resistance to curare like drugs This may partly be due to an especially large ACh release per stimulus and end plate in this muscle by a factor of ten exceeding that of the tibialis anticus muscle It is for such reasons not entirely impossible that even these figures are compatible with the suggestion outlined above that the quantal packet might correspond to only a *fraction* of the transmitter content of a vesicle (granule) but that this fraction might vary from muscle to muscle The packet size might then be set not primarily by the total vesicle content but by the presence of different transmitter pools within the individual vesicle and/or by the number of specific attachment sites present at the area of the nerve membrane which comes in contact with the vesicle (granule)

It is obvious that definite conclusions concerning the detailed arrangement of transmitter release at nerve endings can hardly be drawn at present Nevertheless the present considerations appear to be justified since they may help to shed further light on the problem concerning the size of the quantal packet and the nature of normal transmitter turnover It appears in any case that the present evidence from the muscle vascular bed concerning the *adrenergic* transmission favours the view that only a small fraction of the granule content of NA is released per stimulus This view is as mentioned also in good agreement with a number of *in vitro* and *in vivo* studies (cf Carlsson 1965 Iversen 1967) which suggest that NA is present in different pools within the granules Only a small fraction of the total pool seems to be immediately available for release while the main fraction serves as a storage pool

It has in fact also been suggested that the ACh vesicles in cholinergic nerves may contain ACh in different pools Studying the ACh metabolism in the perfused cat superior cervical ganglion Birks and MacIntosh (1961) have revealed the existence of two rather distinct presynaptic pools of transmitter—a readily releasable

fraction being small compared to a non readily releasable depot fraction It is assumed that these fractions are contained in the synaptic vesicles and that a third fraction of the ACh is contained in the extra vesicular space where it is exposed to hydrolysis by the AChE (cf Eccles 1964) Since the synthesis of ACh is thought to occur within the vesicles the ACh is thus protected from the AChE

In other words the organization of the adrenergic and cholinergic neurons have many features in common The transmitter is synthesized in vesicles (granules) of about the same size Outside the vesicles but within the neuron enzymes for transmitter destruction MAO and AChE respectively are present Within the vesicles the transmitter seems to be held in different fractions one larger depot fraction and one smaller 'readily releasable faction The granules of the adrenal nerve terminals appear to be formed in the cell bodies to be transported down the axons to the nerve terminals where they act as factories for the local NA synthesis (Dahlstrom 1966 Dahlstrom and Haggendal 1966 b) Some observations concerning cholinergic nerve fibres suggest that the situation may be similar to that in adrenergic fibres in these respects also It thus seems as if the ACh manufacturing system is produced in the nerve cell and transported down the axon to the nerve terminals (Bannister and Scrase 1950 Hebb and Waites 1956) Further it has been shown by electron microscopy that apparently cholinergic vesicles accumulate above a constriction in a nerve stem in the same way as is the case with the granules in adrenergic fibres (Dahlstrom and Hokfelt 1966 personal communication)

II Normal routes of elimination of the adrenergic transmitter

While COMT appears to be of great importance in the liver where it is involved in the clearing of the blood stream from such fractions of the adrenergic transmitter that have escaped to the blood this enzyme seems to be of subordinate importance for the transmitter elimination in most tissues (see Introduction) This is in agreement with the present results on the calf muscle preparation MAO has also been discussed as a possible route of elimination of the released adrenergic transmitter but mainly *within* the nerve endings after reuptake through the nerve membrane In fact neither a COMT inhibitor nor a MAO inhibitor significantly increased the NA escape to the blood stream from the stimulated calf preparation Furthermore these enzyme blockers did not retard the relaxation of smooth muscle effectors upon interruption of sympathetic discharge a process that seems to be distinctly prolonged whenever the rate of transmitter elimination is delayed (see e g Figs 2 and 3)

It would appear as if the normal escape of NA released at sympathetic stimulation by means of diffusion from *resting* skeletal muscle to the blood stream may normally account for only some 20-25 per cent of the transmitter released in this tissue This approximate figure is based on the observation that less than 10 ng/min per 100 g of muscle escaped to the blood in this situation while the escape may increase 2-3 fold during exercise hyperemia and 4-5 fold when the highest possible level of

blockade of local elimination mechanisms for NA has been accomplished With exception of states of functional hyperemia earlier observations indicate that the local mechanisms for transmitter elimination can cope efficiently with the rate of NA release but only within the physiological range of sympathetic discharge (Folkow 1952) Therefore clear signs of NA overflow with respect to remote effects on sensitized effectors appear first when this normal range of discharge is surpassed (Celander 1954 Brown and Gillespie 1957, Brown 1965) It may in addition be deduced from the figures of Brown and Gillespie that some 80–90 per cent of the released transmitter in the spleen seems to be locally taken care of presumably by means of the reuptake mechanism These earlier findings are thus in agreement with the present results

The most important local mechanism for adrenergic transmitter elimination thus seems to be the membrane pump Membrane pump blockers alone can as mentioned greatly increase the escape of NA as can also but to a somewhat less extent muscle exercise The NA escape after blockade of the membrane pump seems to be only slightly increased when muscle exercise is superimposed It is not known exactly why exercise alone increases the escape it seems likely that it in some way interferes with the efficiency of the reuptake pump but whether this interference is of a specific nature is not known It may be that several types of changes in the local chemical environment affect the efficiency of the active reuptake mechanism e g hypoxia or metabolite accumulation However the enhanced blood flow *per se* may also contribute a view supported by e g the results of Zimmerman and Whitmore (1967)

The active reuptake mechanism seems to be of importance from several points of view It is thus interesting to note that after membrane pump blockade, the NA release upon continuous sympathetic stimulation at 6 impulses per second sometimes decreased gradually after some 10–15 minutes of stimulation It was further observed concerning the n m response that the typical decline of the effector response seen when supraphysiological stimulation rates are employed tended to occur already at high physiological frequencies after membrane pump blockade These results both suggest that the reuptake may be of considerable importance for the maintenance of a constant transmitter quantity per impulse especially at higher rates of discharge as will be further discussed below Further the results of Brown and Gillespie (1957) and of Zimmerman and Whitmore (1967) indicate that the transmitter quantity released per impulse may rapidly become reduced when the rate of stimulation surpasses some 10 impulses per second At these high rates the reuptake mechanism appears to be unable to cope with the large release

Besides the influence that the reuptake mechanism may have on the economy of transmitter turnover for the adrenergic fibres it is of great importance also for the establishment of a normal autonomic control of the effector cells e g those of the blood vessels The present results (see e g Fig 3) clearly show that a blockade of the reuptake mechanism makes the normally swift and precise neurogenic control

of the vascular bed impossible When the membrane pump is blocked, there remains essentially only the escape to the blood stream and this is far too sluggish to allow for the usually swift adjustments of neurogenically controlled effector cells Adjustments then become slow and retarded transferring the normal rapidly operating neurogenic control to a sluggish hormone like control The consequence for reflex and central adjustments of the cardiovascular system is evident The reuptake mechanism here serves the same purpose as specific cholinesterase for cholinergic neuroeffector function to distinctly delimit the time cour e of the nervous message

As mentioned earlier the capacity of this active elimination mechanism seems to be closely balanced to the transmitter turnover occurring at the normal range of fibre discharge but seems to be overwhelmed if the fibres are stimulated at supra physiological rates A very approximate deduction of the capacity of the NA reuptake mechanism involved in the transmitter turnover may be given It has already been mentioned that some 75–80 per cent of the released transmitter seems to be reuptaken during muscle rest at a sympathetic discharge rate of 6 impulses per second Further, there is little evidence of any major increase of transmitter over flow in resting tissues until frequencies beyond 10 impulses per second are reached Lastly the present estimations suggest that each impulse releases some 0 15 ng NA in 100 g of skeletal muscle It then follows that the maximal capacity of the reuptake pump of the adrenergic fibres during discharge would correspond to some 70 ng of NA per minute per 100 g of skeletal muscle (0 15 × 10/sec × 60/sec × 80 per cent) This very approximate figure for transmitter reuptake which thus applies mainly to the neuroeffector junctions during fibre activity can hardly be obtained by measurements of the uptake of exogenous NA since it appears that the entire fibre membrane allows for uptake when NA is diffusely administered via the blood stream Furthermore the active uptake may be more efficient during fibre rest It has been observed that sympathetic stimulation in reserpinized animals where no transmitter is released decreases the uptake of exogenous NA and the more so the higher the impulse frequency This may be due to diminished uptake during the period of membrane depolarisation (Haggendal and Malmfors 1967) It is possible that such a mechanism explains why the membrane pump is unable to cope efficiently with the released transmitter when 'supraphysiological stimulation rates are employed

A membrane pump blockade alone hardly leads to excess accumulation of NA in the blood as revealed by the fact that even a sensitized denervated nictitating membrane was not contracted at the NA concentrations in the blood that were reached after reuptake blockade This was further obvious from the observation that the NA arterial blood levels were never markedly raised The explanation seems to be that especially liver COMT is capable of keeping the blood concentration of NA fairly low in this situation also When COMT was blocked on the other hand it was observed that the arterial NA concentration gradually rose to high levels

III Availability of the transmitter stores

There is at present considerable experimental evidence to suggest that only a minor fraction of the total NA store within the adrenergic nerve terminals is immediately available for release (cf Carlsson 1965 Iversen 1967) The present results lend further support to such a view and may also provide some semi quantitative figures concerning the size relationship between the immediately available store and the main store for NA

As mentioned in section I, the quantity of NA released per stimulus at physiological discharge rates corresponded to only some 2×10^{-5} of the total store in the calf muscle preparation *If the total store were readily available for release it* would with an unchanged NA amount per stimulus last for as much as 90 minutes at a maximal physiological discharge of 8–10 impulses per second no account then being taken for NA synthesis However adrenergic neuroeffectors like the n m (Fig 2 A) seem to fail in maintaining a constant transmitter quantity per stimulus even after a few minutes if stimulation is performed at definitely supra physiological rates i e above 12–14 impulses per second The same phenomenon has been repeatedly observed for the blood vessels also During the first few minutes of a stimulation period this gradual failure is however preceded by an excess NA release with considerable local accumulation and subsequent overflow (see also Folkow 1952 Celander 1954) These circumstances strongly suggest as mentioned in section II that the reuptake mechanism cannot cope with the excess release in this situation A markedly increased loss of transmitter from the nerve terminals will then occur which may be of direct relevance to the subsequent decline of NA release per stimulus Such a gradual decline usually appeared to occur when the NA turnover per *minute* exceeded some 1 5–2 per cent of the expected total store Thus during continuous stimulation at 20 impulses per second the effector responses revealed such a decline already after 2–3 minutes in most cases In this situation it may be expected that nearly half the released NA amount will re enter the nerve terminal since the reuptake mechanism appears to be able to cope with the major part of the release up to some 10 impulses per second It follows that a gradual failure of transmitter release per stimulus may become evident even when the total loss of NA during continuous fibre activity corresponds to only *some 2–3 per cent of the total store to judge from the present figures for NA release and content*

These results are in agreement with earlier observations suggesting a gradual decline of transmitter release per stimulus both at pre and postganglionic nerve terminals whenever supraphysiological stimulation rates are applied to sympathetic nerve fibres (Folkow 1952) On the whole such a principle appears to be valid for autonomic nerve fibres in general (e g Perry 1953 Kewenter 1965) It may further be deduced from the data of Brown and Gillespie (1957) and Haefely Hurlimann and Thoenen (1965) that the NA release per stimulus from the cat spleen seems to be largely constant or even to increase somewhat up to discharge rate of about

10 impulses per second only to fall rapidly at higher, supraphysiological rates of stimulation

The present results also suggest however that the balance between release and uptake of NA may be maintained almost indefinitely as long as the NA release per *minute* is below some 1–1 5 per cent of the total store a level reached first at an impulse rate of about 10 per second This would correspond to a release per *minute* of some 80–100 ng (0 15 ×10×60) in 100 g of muscle from a total NA store of about 8000 ng This figure for well sustained maximal physiological release per minute corresponds well with the earlier mentioned slightly lower figure for maximal reuptake capacity of the adrenergic transmitter deduced to be some 70 ng per minute for the adrenergic nerve terminals in 100 g of skeletal muscle

It is likely that release and reuptake nearly balance each other in most normal situations thus keeping the evidently small NA store that is readily available for release largely constant NA synthesis may be expected to compensate efficiently for the slight dysbalance between release and uptake which is the result of the usually small fraction of NA that escapes to the blood stream (some 20–25 per cent during muscle rest) To judge from other studies the NA synthesis per minute appears to correspond to up to 0 25 per cent of the total store (for ref see Iversen 1967) For 100 g of skeletal muscle this would mean a NA synthesis per minute of some 20 ng This amount appears to be more than enough to cover the losses to the blood stream in most physiological situations where only episodically the discharge rate seems to reach or surpass some 5–6 impulses per second (Folkow 1952)

When the reuptake of NA is blocked so that essentially the total NA release escapes to the blood stream the situation may become more critical with increased risks for exhaustion of the readily releasable NA store In this situation a gradual decline in the release per stimulus sometimes appeared to occur within relatively few minutes when stimulation rates corresponding to the higher physiological discharge range i e 8–10 impulses per second, were employed (e g Fig 2 B)

When on the other hand the same phenomenon was studied at a stimulation rate of 6 impulses per second and now in terms of the NA escape from the calf muscles to the blood stream a relatively constant release per stimulus could usually be maintained for about 10–15 minutes though a gradual decline could subsequently follow With this smaller NA escape per minute it is possible that the limited capacity for NA synthesis may more efficiently counteract the gradual decline of the readily releasable NA store As discussed earlier the NA synthesis during fibre rest or low activity is likely to be of the order of some 20 ng per minute in 100 g of skeletal muscle In fact this amount may be expected to increase somewhat in the actual situation since intense fibre activity will probably facilitate the synthesis process For example Fredholm and Sedvall (1966) observed a more than 2 fold increase of NA synthesis in the salivary gland at high rates of sympathetic stimulation Suppose that the NA synthesis is about doubled during the stimulation period and that this new

of a varicosity should correspond to the quantal packet of the adrenergic transmitter

5 The mentioned average NA fraction being released per impulse from the individual nerve fibre varicosity corresponds to approximately 400 NA molecules while a granule appears to contain about 15 000 NA molecules These 400 NA molecules will create an average concentration in the junction gap of the order of 0 5–1 μg/ml

6 This NA release per impulse from the average fibre varicosity may well represent the sum of several NA quantal packets It is therefore possible that the ultimate packets of NA which seem to be released at random even during fibre rest to produce subthreshold miniature potentials are made up of *less* than 400 NA molecules Such ultimate packets may then emanate from different granules

7 Some possible mechanism(s) by which the suggested quantal packet for NA may be separated from the total content of a varicosity granule are briefly outlined

8 Some similarities and dissimilarities between the adrenergic and the cholinergic *transmission mechanisms are pointed out It is discussed whether the acetylcholine* (ACh) quantal packet might also correspond to only a relatively small fraction of the transmitter content of one ACh vesicle

9 The present results and considerations are in agreement with earlier results which mainly on a basis of pharmacological evidence suggest that the major part of the NA content of adrenergic nerve fibres forms a storage pool while only a minor fraction in the order of a few per cent is immediately available for release

10 This immediately available NA fraction of the individual granule appears to be fairly rapidly exhausted if the fibres are driven at supraphysiological frequencies Further if the NA reuptake mechanism is blocked exhaustion of this readily available *transmitter fraction seems to be more rapid and may then even occur at dis*charge rates within the higher physiological range However normally the active NA reuptake combined with NA synthesis may easily keep this readily available store largely constant

II *The elimination of the adrenergic transmitter*

1 In conformity with earlier investigations the present results suggest that by far the most important route for local elimination of the transmitter at the vasoconstrictor nerve endings is constituted by the active reuptake mechanism In *the calf muscle preparation this mechanism seems to account for 70—80 per cent* of the released amount perhaps even more at least during tissue rest The major part of the remaining NA fraction escapes to the blood stream while enzymatic destruction at the neuroeffector junction seems to be largely negligible

2 Evidence is presented to indicate that the maximal capacity of the NA reuptake mechanism at the neuroeffector junctions in 100 g of muscle is of the order of 70 ng per minute This may therefore account for reuptake of the major part

of the release occurring during physiological conditions It could be deduced from figures in the literature concerning the capacity of NA synthesis in various tissues that the NA synthesis in the calf muscles may be more than enough to cover the expected deficit between release and reuptake over the entire physiological range of sympathetic discharge rate

3 The reuptake mechanism may be considerably interfered with during muscle exercise since more NA then escapes to the blood stream This increased escape seems to be due at least in part to the improved diffusion conditions as a result of the increased blood supply *It is however possible that some secondary change* in the chemical environment may more specifically interfere with the active reuptake

4 It was shown that the active NA reuptake is all important for allowing the normally swift neurogenic adjustments of the adrenergically innervated effectors *If this reuptake mechanism fails* transmitter elimination by means of diffusion is such a slow process that the nervous control would resemble the retarded adjustments induced by hormones This would make the rapid reflex adjustments that are needed e g for the efficient control of the cardiovascular system impossible

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HYDROXYLATION OF PROLINE IN THE BIOSYNTHESIS OF COLLAGEN

AN EXPERIMENTAL STUDY WITH CHICK EMBRYO AND GRANULATION TISSUE OF RAT

BY

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SYMBOLS AND ABBREVIATIONS

Hypro	hydroxyproline
mRNA	messenger RNA
sRNA	soluble (transfer) RNA
S	Svedberg unit

Turku Kirjapaino Polytypos 1968

CONTENTS

INTRODUCTION

Collagen is the main fibrous protein in connective tissue and the most abundant single protein in body. The ability of fibroblasts to synthesize large quantities of collagen on demand is a special feature of connective tissue. The biosynthesis of collagen offers an advantageous model for studies on common aspects in biosynthesis of proteins because it contains two internal labels hydroxyproline and hydroxylysine.

Most studies on the biosynthesis of collagen are based on the formation of hydroxyproline. The classic observations of Stetten and Schoenheimer (1944) and Stetten (1949) indicated that proline is a precursor of hydroxyproline in collagen. This has since been confirmed by a number of investigators. Similar relationship has been established between lysine and hydroxylysine (van Slyke and Sinex 1958). These circumstances are unique for the biosynthesis of collagen.

Proline is a common amino acid in proteins on the contrary to hydroxyproline found in significant amounts only in collagen. This suggest that the proline destinated for collagen hydroxyproline differs from the proline going to other proteins. The most plausible explanation is that the hydroxylation of proline occurs in some intermediate, in a precursor of collagen. The possibility to find such a precursor has stimulated many investigators and controversial reports are numerous.

The insufficient knowledge about the nature of the intermediate of collagen hydroxyproline has limited the studies on the mechanism of hydroxylation. During the last years considerable progress has been made in this field providing an additional aspect for studies on the regulation of collagen biosynthesis.

REVIEW OF LITERATURE

The nature of the precursor for collagen hydroxyproline

The biosynthesis of collagen differs from the conventional scheme for the synthesis of protein in respect that hydroxyproline and hydroxylysine are not incorporated in collagen Stetten and Schoenheimer (1944) and Stetten (1949) showed that free proline could serve as a precur or of collagen hydroxyproline but free hydroxyproline did not incorporate in collagen *in vivo* The e observations have been verified both *in vivo* and *in vitro* studies by several investigators (*e g* Smith and Jackson 1957 Wolf and Berger 1958 Green and Lowther 1959 Peterkofsky and Udenfriend 1961) The same relation hip was established b tween lysine and hydroxylysine (*e g* Sinex and van Slyke 1955 Piez and Likins 1957, van Slyke and Sinex 1958 Kao and Boucek 1958*a* & *b* Sinex van Slyke and Christman 1959 Popenoe and van Slyke 1962) Mitoma *et al* (1959) reported however that labeled hydroxyproline incorporated into collagen in chick embryos but the specific activity of bound hydroxyproline was only one tenth of that observed when labeled proline was employed as a precursor

Inve tigations for the bio ynthesis of collagen are generally ba ed on the appearance of protein bound hydroxyproline or hydroxylysine It is obvious however that two proces es peptide synthesis and hydroxylation are involved in conversion of proline and lysine to protein bound hydroxyproline and hydroxylysine re pectively Hydroxylation of prolin and lysine can occur either at the stage of free proline or lysine prolyl or lysyl adenylate prolyl or lysyl-sRNA or ribosomal peptide bound proline or lysine or after the release of peptide chain from ribosomes The parent amino acid destinated for the hydroxylation must occur in a sequestered or bound form that distinguishes it from the proline or lysin which is incorporated into collagen and other proteins but remaind unhydroxylated

Two hypotheses have been propo ed as to the site at which proline is hydroxylated (1) hydroxylation occurs prior to the formation of peptide linkage in which case it is suggested that activated proline or prolyl sRNA are the immediate precur ors (2) proline is hydroxylated after it is incorporated into peptid or protein linkages

Hydroxylation before the peptide stage Robertson Hewitt and Herman (1959) Robert on and Hewitt (1961) report d that a corbic acid added to a deficient tissue increased the pecific activity of collagen hydroxyproline ari ing from proline The observation that the rate of proline incorporation into granuloma proteins by scorbutic guinea pigs was not reduced to the ame degree as the formation of collagen bound hydroxyproline This led to a concept that hydroxylation of proline occurs at the stage prior to incorporation into collagen (Stone and Meister 1962*a*) Because no

evidence for an accumulation of a proline rich collagen precursor has been demonstrated, the existence of two separate proline pools destinated for collagen was suggested (Meister, Stone and Manning 1964 Robertson 1964)'

Daughaday and Mariz (1962*a*) inculated fragments of rat s costal cartilage with labeled proline They observed that free hydroxyproline, having a specific activity of 20 times that of hydroxyproline of soluble collagen, was released in the medium They concluded that free hydroxyproline arised from an activated hydroxyproline intermediate, which exist before the stage of peptide synthesis Levine (1962) isolated a labeled nondialyzable fraction from the medium where minced rat skin was incubated with ^{14}C proline The specific activity of hydroxyproline was 8 to 12 fold that of proline Treatment of the fraction by autoclaving at pH 10 or with ribonuclease, resulted in the release of radioactivity which could be removed by dialysis Therefore he suggested that this fraction represents a peptide precursor of collagen possibly bound to RNA Manner and Gould (196 1963) isolated labeled hydroxyprolyl sRNA from chick embryos labeled with ^{14}C proline *in vivo* Similarly they found ^{14}C hydroxy prolyl sRNA when chick embryo sRNA was incubated with C proline in a cell free system from chick embryo They concluded that proline can be converted to hydroxy proline without prior incorporation of the proline into protein Coronado Mardones and Allende (1963) reported synthesis of labeled hydroxyprolyl RNA and hydroxy lysyl sRNA in a system containing C proline or C lysine activating enzyme and sRNA from chick embryo In anaerobic conditions only C prolyl sRNA was formed. When the isolated ^{14}C prolyl RNA was reincubated aerobically in the same system a labeled hydroxyprolyl sRNA was formed (Coronado *et al* 1964) Jackson Watkins and Winkler (1964) isolated highly labeled hydroxyprolyl sRNA from chick embryo and wound granulation tissue labeled with tritiated proline *in vivo*

Hydroxylation of proline in peptide stage or later Stetten (1949) first suggested that hydroxylation of proline takes place in a peptide Fraser and Klass (1963) found that partly purified prolyl sRNA synthetase from rat liver did not catalyze the formation of hydroxyprolyl sRNA The same preparation promoted slight hydroxy proline dependent exchange between ATP and ^{32}P pyrophosphate Peterkofsky and Udenfriend (196) had reported that in cell free system from chick embryo amino acid activation occurred with L proline but not with L hydroxyproline On the contrary Jeffrey and Martin (1966*a*) found that in soluble fraction from chick embryos and tibiae the hydroxyproline dependent ATP P pyrophosphate exchange ranged from 30 to 50 % of the proline dependent exchange They suggested in view of the work by Norris and Berg (1964) that in the transfer of hydroxyproline from the adenylate to sRNA the hydroxyproline adenylate enzyme complex becomes unstable and dissociates

Peterkofsky and Udenfriend (1963*a* *b*) presented evidence against the possibility that proline is hydroxylated at the level of sRNA They observed in a cell free system from chick embryos a significant incorporation of C proline into protein bound proline and hydroxyproline associated with microsomes The incorporation of proline to protein bound form started in the beginning of the incubation but significant amounts of labeled hydroxyproline were not detected until after an incubation period of 30 min. Puromycin and ribonuclease inhibited the incorporation of proline into protein bound hydroxyproline completely when added in the beginning of incubation However when these were added at the end of the 30 min lag phase puromycin

inhibition was negligible and ribonuclease inhibition was considerably reduced During the lag period the system did not require oxygen They concluded that substrate for hydroxylation is a proline rich polypeptide which is either bound or loosely associated with microsomes Urivetzky Frei and Meilman (1965) employed cell free systems from chick embryo and found them to be active in incorporating free C proline or C prolyl sRNA into ribosomal protein Aerobic conditions enhanced the formation of peptide bound C hydroxyproline particularly when C-prolyl RNA was used as a precursor In both cases the incorporation of hydroxyproline lagged behind that of proline Several laboratories have also reported unsuccessful attempts to isolate hydroxyprolyl sRNA from chick embryos (Lukens 1965 Manning and Meister 1966 Udenfriend 1966)

Suggestive evidence for synthesis of a peptide precursor of collagen can now be found in literature As mentioned earlier Stone and Meister (196 a) reported that in minced scorbutic granuloma the incorporation of labeled proline was comparable to that in normal granuloma The synthesis of hydroxyproline was however negligible This dissociation could be explained by a formation of a hydroxyproline deficient precursor of collagen Similarly Urivetzky Kranz and Meilman (1963) found that a cell free system from rabbit skin incorporated labeled proline into ribosomal protein No conversion of proline to hydroxyproline was observed The synthesis of anomalous collagen which contained a higher proportion of proline than normally, was suggested by Hurych and Chvapil (1965a) They studied the effect of various chelating agents on the biosynthesis of collagen The most effective of the tested ones was $\alpha\alpha$-dipyridyl The synthesis of hydroxyproline was negligible in skin slices of chick embryo incubated in the presence of this chelator The specific activity of proline in the inhibited sample was doubled in comparison to that of the control sample

Mechanism of the hydroxylation of proline

The oxygen in the hydroxyl group of hydroxyproline is derived from atmospheric oxygen and not from water (Fujimoto and Tamiya 196_ 1963, Lamport 1963 Prockop Kaplan and Udenfriend 196? 1963) These studies provided evidence that proline is hydroxylated by an oxygenase mechanism Proline is converted to hydroxyproline by a direct displacement type of hydroxylation where one hydrogen is released from carbon atom in position 4 of the pyrrolidine ring (Ebert and Prockop 196_ Konno and Tetsuka 196 Lamport 1964 Prockop Ebert and Shapiro 1964) Stone and Meister (1962 *b*) reported however the loss of two hydrogen atoms during the conversion of proline to hydroxyproline

Direct oxygenase mechanism in hydroxylation of proline was confirmed by Fujita *et al* (1964) They prepared *cis* and *trans*-4 H L-proline and observed that only the tritium at *trans* position to carboxyl group was lost during the synthesis of collagen hydroxyproline in chick embryos The mechanism of the hydroxylation of lysine is most likely to be similar to that of proline (Popenoe Aronson and van Slyke 1965, 1966)

Proline can be hydroxylated also by a non enzymatic system (Chvapil and Hurych 1959 Hurych and Chvapil 1969) and by horseradish peroxidase (Yip 1964) The significance of these types of reactions in the biosynthesis of collagen remains questionabl

Model systems for studies on collagen synthesis *in vitro*

Many experimental systems have been introduced for the studies on the biosynthesis of collagen *in vitro*. The work of Green and Lowther (1959) in which carrageenin granuloma slices were employed as a collagen synthesizing tissue, can be considered as a classic one in this field. Slices of collagen synthesizing tissues of various animals have been employed, *e.g.* rat uterus (Kao *et al.* 1962), rat cartilage (Daughaday and Mariz 196 *a b*), rat bone (Davis, Holmes and Johnson Jr. 1962; Flanagan and Nichols Jr. 1962), chick embryo (Lukens 1965), chick embryo cornea (Coleman, Herrmann and Bess 1965), chick embryo skin (Hurych and Chvapil 1965), rabbit skin (Bekhor and Bavetta 1965). Chick embryo cartilage has been used in tissue culture (Biggers 1960; Jeffrey and Martin 1964, 1966*a b*). Differentiated embryonic chondrocytes were employed by Prockop, Pettengill and Holtzer (1964), Birge and Peck (1966) and osteoblasts by Jackson and Smith (1957). Green and Goldberg (1963, 1964) have studied the capacity of various fibroblast lines to synthesize collagen in cell culture.

Comparison of the capacity of various tissues to synthesize collagen is not possible to perform in detail because the experimental conditions vary a great deal. In most investigations the main attention has been paid to the comparison of the control and test samples. Further, the results about the incorporation of labeled precursors and synthesis of labeled hydroxyproline are expressed in μmoles, cpm, dpm per incubation periods employed, per hour and further per wet or dry weight, mg of cell nitrogen, mg of DNA, *etc.*

A cell free system for studying biosynthesis of collagen has been presented by Peterkofsky and Udenfriend (1936*a b*) employing chick embryos and by Urivetzky, Kranz and Meilman (1963) using rabbit skin. Although the activity of these systems is low as compared with that of whole cell, valuable information has been achieved on the mechanism of collagen synthesis.

Granulation tissue offers a medium for studies on the limiting factors in the biosynthesis of collagen. A granuloma induced by viscose cellulose sponge has been found well suited for studies on various aspects of granulation tissue (Viljanto 1964; Kulonen 1965). In contrast to other types of experimental granulomas, *e.g.* carrageenin granuloma, where the stage of development of granulation tissue is not even throughout the tumor, the sponge granuloma has the advantage of being homogenous and can be easily separated from the surrounding tissue. The age of the granuloma correlates well with the developmental stages of granulation tissue from one experiment to another, thus providing a possibility for comparison of granulomas in various phases of growth. The synthesis of collagen starts on about the 4th to 5th day after implantation of sponge pieces as judged by the incorporation of proline and synthesis of hydroxyproline *in vitro*. The rate of collagen synthesis increases rapidly and reaches its maximum on the 18th to 21st day followed by a rapid fall. At the age of 40—50 days the synthesis of hydroxyproline has already practically ceased (Lampiaho and Kulonen 1967). This age-depending rate of collagen synthesis offers an approach to studies on the regulation of collagen synthesis.

Effects of puromycin and chloramphenicol on protein synthesis

Puromycin Puromycin consists of an aminonucleoside linked to the amino acid p methoxyphenylalanine It can be regarded as an analogue of adenosyl phenylalanine specifically of the terminal group of the phenylalanyl sRNA The antibiotic substitutes for aminoacyl sRNA and attaches by its amino group to the end of a growing polyp ptide chain The carboxyl group of puromycin is not available for an additional peptide bond and the peptide chain can not grow further Relatively short chain each carrying a puromycin molecule are then relea ed from ribosomes (*e g* Yarmolinsky and de la Haba 1959 Allen and Zamecnik 1962 Allen and Schweet 1962 Nathans *et al* 196?*a*) The puromycin inhibition is reversible as indicated in studies by Morris and Schweet (1961) William on and S hweet (1964) Puromycin has b en used as a tool for studying protein synthesis both *in vivo* and *in vitro* The e studies are reviewed by Darken (1964) and by Nathans (1964)

Manner and Gould (1962) reported that collagen synthesis in chick embryos was almost completely inhibited by puromycin In further experiments they showed that the incorporation of labeled proline and formation of hydroxyproline in soluble and insoluble collagen was markedly inhibited The formation of free hydroxyproline however was considerably less inhibited (Manner and Gould 1963) In granulation tissue the synthesis of collagen was decreased by puromycin (Kulonen *et al* 1964 Meister Stone and Manning 1964 Manning and Meister 1966) and in embryonic chick tibia grown in tissue culture (Jeffrey and Martin 1964 and 1966*b*) Juva and Prockop (1964) found that the antibiotic inhibited the synthesis of labeled hydroxy proline to a greater extent than it inhibited the incorporation of labeled proline into protein by embryoni cartilage They also found that nondialyzable polypeptides rich in labeled proline accumulated in the soluble fraction of the tissue Similar results were reported by Lukens (1965)

Chloramphenicol Chloramphenicol has been shown to be a potent inhibitor of protein synthesis *in bacteria* The mode and site of the effect are somewhat uncertain however Studies on the mechanism of inhibition in microbiological cell free systems have indicated that inhibition occurs at a stage sub equent to the attachment of amino acids to sRNA and at a site related to the ribo omal a embly of amino acids on template RNA (*e g* D Moss and Novelli 1956 Lacks and Gross 1959 Nathans and Lipman 1961 Nathans *et al* 196 *b* Pendi and Ochoa 1962) An accumulation of RNA resembling mRNA was d monstrated in bacterial cells by Hahn and Wolfe (1962), Midgley and McCarthy (1962) On the basis of these observation it was suggested that chloramphenicol might interf re with the attachment of mRNA to ribosomes (Bendi and Ochoa 1962) Vazquez (1964) reported that chloramphenicol binds to the 50 S ribo omal subunit Wolfe and Hahn (1965) confirmed this ob ervation and found that the binding was rever ible and it was not affected by the presence of polyuridylic acid. Evidence that peptide linking step is possibly involved has been reported by Julian (1965)

Animal cells are considered more resistant again t the effect of chloramphenicol This is ac ounted for by differences in the mechani m of protein synthe is in mammalian and bacterial cells However proce ses involving cell differentiation uch as antibody synthesis and reticulocyte formation are inhibited by chloramphenicol (Weisberger and Wolfe 1964) Chloramphenicol has no effect on protein synthesis in cell free

systems of animal origin. Significant inhibition in mammalian cell free systems was obtained however, when protein synthesis was stimulated by adding template RNA (Weisberger and Wolfe 1964, Wolfe and Weisberger 1965). Their observations are in agreement with the suggestion that mammalian cells are susceptible to chloramphenicol at the time when new mRNA is being attached to ribosomes. The effect of chloramphenicol on protein synthesis has been reviewed by Weisberger and Wolfe (1964) and by Schweet and Heinz (1966).

OUTLINES OF PRESENT INVESTIGATION

When the experiments of this investigation were started in 1964, a controversy about the site for the hydroxylation of proline existed The concept that the hydroxylation occurs in prolyl sRNA was predominant

In the preliminary experiments it was observed a disproportional effect of puromycin on the synthesis of collagen and of other proteins suggesting that the hydroxylation of proline occurs in a peptide precursor of collagen (Juva and Prockop 1964) The present investigation is a recapitulation and supplemental continuation of the investigations by Juva and Prockop (1965 1966*a* *b* *c*), Prockop and Juva (1965*a* *b*)

This study resolves itself into three parts

1 Chick embryo tibia was tested as a model system to study the bio synthesis of collagen *in vitro* The effect of puromycin on the synthesis of collagen and other proteins was studied and comparation was made with the effect of chloramphenicol

2 The synthesis and characteristic of the peptide precursor of collagen hydroxyproline were studied The enzymatic system required for the hy droxylation of proline in the polypeptide precursor of collagen was partially characterized

3 The synthesis of the polypeptide precursor of collagen and the hy droxylating activity of rat granulation tissue of different age were demon strated

MATERIAL AND METHODS

Incubation of chick embryo tibiae

Embryonated chicken eggs 0 to 9 days old were purchased from local hatcheries They were incubated in a moist atmosphere at 38° until used During the course of experiments it was observed that 10 day old tibiae had a thin osseous collar round the diaphysis firm enough to ensure successful dissection without greater damage to tissue Therefore, embryos of this age were chosen for most of the experiments presented Cells in four stages of differentiation are observed in tibiae of 10 day old chick embryos small round cells in the epiphyseal zone flattened cells in the adjacent region enlarging cells in the next region and terminally hypertrophied chondrocytes in the middiaphysis (Fell 1925)

All instruments and glassware were sterilized by autoclaving for 30 min at 120° The embryos were removed from the eggs and the legs were cut off with forceps The legs were placed on petri dishes which contained the incubation medium and the tibiae were prepared by microscopic dissection with sterile precautions

The incubations were carried out employing a modified phosphate saline medium 2 A of Krebs (Krebs 1950) unless indicated otherwise The organic constituents were omitted except glucose Penicillin 35 I U /ml and streptomycin 35 μg/ml were added to the medium which was sterilized by filtering through the bacterial filter (Millipore GSWP 04700 0 22 μ Millipore Filter Co Bedford Mass, U S A) The cultures of the medium in thioglycolate medium indicated that the samples were free of bacterial contamination after incubation

The tibiae were placed in round bottom culture tubes containing 2 7 ml of medium Puromycin and chloramphenicol dissolved in the medium were added into the tubes in a volume of 0 2 ml After a preincubation period of 20 min at 37° isotopes were added in 0 1 ml of the medium

In order to prepare matched samples, one tibia from an embryo was used as a control and the other as a test In most experiments four tibiae

from two embryos were crosspaired to increase the observed isotope incorporation The tubes were covered with cotton plugs and incubated in a metabolic shaker (New Brunswick Scientific Co New Brunswick N J USA) in a moist atmosphere of 95 % oxygen and 5 % carbon dioxide at 37°

Incubation of granuloma slices

Granulomas were produced as described by Viljanto (1964) Male albino rats of Wistar strain, weighing 200—240 g at the day of implantation were used throughout the experiments Under ether anaesthesia 4 pieces of viscose cellulose sponge (size 10×10×20 mm weight 60—90 mg) were implanted symmetrically under the dorsal skin of the rats The pieces were sterilized by boiling in 0 15 M sodium chloride solution for 10 min After desired periods the rats were killed by a blow on the neck and the pieces with the ingrown granulation tissue were dissected and immersed in ice cold 0 15 M sodium chloride solution

For the studies on the synthesis of collagen precursor, 3 granulomas were cut in 0 5 mm thick slices with a Stadie Riggs microtome The slices (10—12 g wet weight) were incubated in 50 ml Erlenmeyer flasks contained 15 ml of Krebs 2 A solution (Krebs 1950) Test samples contained α α dipyridyl in the final concentration of 1×10^{-4} M After a preincubation period of 20 min 5 μCi of L proline ^{14}C were added to the samples and the incubation was continued in air at 37° for 3 h

Processing of the samples

Tibiae After the incubation tibiae were homogenized at 1500 or 4500 rev/min for 2 min A glass Teflon homogenizer of Potter Elvehjem type was used (size A clearance 0 1—0 15 mm Arthur H Thomas Co, Philadelphia Pa USA) and kept in an ice bath during the homogenization Water was used as a homogenizing medium unless indicated otherwise The homogenate was dialyzed with 100 mg of L proline overnight against running tap water A few drops of toluene were added inside the bags to prevent bacterial growth

After dialysis equal volumes of concentrated hydrochloric acid (ACS reagent Fisher Scientific Co) were added to the retentates and the samples were hydrolyzed at 120° for 12—16 h The hydrolyzates were evaporated to dryness *in vacuo* The residues were dissolved in 4 0 ml of water and aliquots were taken for the assay of total radioactivity and labeled hydroxyproline

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Prockop and Udenfriend (1960) described a method for the assay of hydroxyproline which can be applied directly to crude tissue or protein hydrolyzates containing less than 0 01 % hydroxyproline Prockop Udenfriend and Lindstedt (1961) adapted this method to the determination of specific activity of radioactive hydroxyproline In these methods imino acids are oxidized with chloramine T Interfering substances as well as the oxidation product of proline Δ^1 pyrroline are first removed by extractions with chloroform or toluene The oxidation product of hydroxyproline is then converted to pyrrole which is extracted with toluene In the modification of Peterkofsky and Prockop (1962) known amounts of carrier proline and hydroxyproline were added to ensure consistent recoveries of the radioactivity from both imino acids The recovery of proline was determined by comparison with a radioactive standard and the recovery of carrier hydroxyproline was determined colorimetrically The interference from radioactive proline in the final toluene extract contained pyrrole from hydroxyproline was reported to be 0 05 % During the course of this investigation it became obvious that the interference from radioactive proline was considerably higher and variable Especially in samples containing an excess of radioactive proline compared with radioactive hydroxyproline erroneously high values for hydroxyproline were observed Therefore a modified procedure for the assay of radioactive hydroxyproline was developed (Juva and Prockop 1966*a*)

Reagents

Chloramine T analytical grade (Eastman Organic Chemicals Rochester N Y U S A) 0 [illegible] M aqueous solution was prepared immediately before use

Sodium pyrophosphate ACS reagent (Fisher Scientific Co) 0 [illegible] M buffer adjusted to pH 8 0 with hydrochloric acid

Sodium thiosulphate ACS reagent (Fisher Scientific Co) 3 6 M aqueous solution stored under toluene at room temperature

Tris buffer primary standard (Fisher Scientific Co) 1 0 M buffer adjusted to pH 8 0 with hydrochloric acid

Silicic acid Bio Sil HA (Bio Rad Laboratories Richmond Calif U S A)

p Dimethylaminobenzaldehyde practical grade (Eastman Organic Chemicals), was recrystallized twice from hot absolute ethanol The solution was prepared as described by Prockop Udenfriend and Lindstedt (1960) and stored at − [illegible]0

Pyrrole practical grade (Fisher Scientific Co) was redistilled A 0 01 mM standard solution was prepared by the dilution of freshly distilled pyrrole with toluene and stored at − [illegible] It was found to be stable for several months based on the chromogen formation with p dimethylaminobenzaldehyde

Procedure

An aliquot containing less than 80,000 dpm of ^{14}C or less than 150,000 dpm of ^{3}H was taken for the assay of radioactive hydroxyproline. The aliquot was placed in a screw capped test tube (Kimble 45066-A) and 1.0 mg L-proline (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.) and 2.0 mg of L-hydroxyproline (Nutritional Biochemicals Co.) were added as concentrated solutions. The pH was adjusted to 8.0 with 1.0 N sodium hydroxide and checked with a pH meter (Radiometer, Copenhagen, Denmark). The volume was made up to 8 ml with water. Six ml of 0.2 M sodium pyrophosphate buffer, pH 8.0, was added followed by 1.0 ml of 0.2 M chloramine-T solution. After oxidation at room temperature for 20 min the reaction was stopped by adding 6.0 ml of 3.6 M sodium thiosulphate solution. The pH was adjusted to a faint pink color of phenolphthalein. Then 4.0 ml of 1.0 M tris buffer, pH 8.0, was added, and the sample was saturated with an excess of sodium chloride (ACS reagent, Fisher Scientific Co.). Ten ml of toluene was added, the tube was sealed and shaken vigorously for 5 min. The tube was centrifuged at 600×*g* for 5 min, and the toluene layer was carefully removed. The tube was sealed again and heated in a boiling water bath for 25 min. The sample was cooled to room temperature and sodium chloride was added, if necessary, to keep the solution saturated. Exactly 12.0 ml of toluene was added and the sample was shaken again for 5 min. After centrifugation 10.0 ml of the toluene layer was placed on a silicic acid column. It was prepared by pouring a slurry of 1.5 g of silicic acid in 5.0 ml of toluene into a glass column (size 10×30 mm) with a sintered glass disk. To prevent clogging, a thin layer of acid washed sea sand was placed on the disk. The column was eluted with 5.0 and 10.0 ml of toluene successively. A 20.0 ml aliquot of the pooled effluent (25 ml) was transferred to a counting vial with 1.0 ml of phosphor solution for the assay of radioactivity. For the colorimetric assay of recovered pyrrole, a 0.1 ml aliquot was diluted with 5.0 ml of toluene. Two ml of Ehrlich's reagent was added and mixed rapidly with a Vortex mixer (Scientific Industries Inc., Springfield, Mass., U.S.A.). The color was allowed to develop in the dark at room temperature for 30 min, and the absorbance was measured at 560 nm against a reagent blank employing a Beckman B spectrophotometer (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). The amount of pyrrole in the sample was calculated by reference to pyrrole standards of 0.02 and 0.04 µmoles, which were developed at the same time. A control containing 1.0 mg L-proline, 2.0 mg of L-hydroxyproline and 80,000 dpm of L-proline-^{14}C or 150,000 dpm of L-proline-3,4-^{3}H was run together with each series of samples.

Calculations Observed cpm in the final toluene extract were converted to dpm of radioactive hydroxyproline in the aliquot taken for assay by correcting for the background counts for the aliquots, for the % recovery of carrier hydroxyproline as pyrrole in the chemical assay, and for the loss of isotopic label in the conversion of hydroxyproline to pyrrole The following formula was used to calculate the amount of hydroxyproline ^{14}C in the aliquot oxidized from the observed cpm in final solution of pyrrole ^{14}C

$$\text{observed cpm} \times \frac{100}{E} \times \frac{25}{20} \times \frac{5}{4} \times \frac{100}{R} = \text{dpm}$$

where E is the efficiency of the counting system 25/20 is the correction for aliquot of effluent which was counted, 5/4 is the correction for the loss of the carboxyl carbon in the conversion of uniformly labeled hydroxyproline ^{14}C to pyrrole ^{14}C and R is the % recovery of hydroxyproline as pyrrole in the chemical assay Total dpm of hydroxyproline-^{14}C in the sample was obtained by correcting for the aliquot which was taken for oxidation When hydroxyproline 3 4 ^{3}H was assayed the correction factor for the loss of label during the procedure was 3/2 In samples where the total incorporation of L proline 3 4 ^{3}H into protein was compared to the incorporation into hydroxyproline ^{3}H however a correction factor 4/2 was used (Prockop Kbert and Shapiro 1965) The recovery of hydroxyproline as pyrrole (R) was calculated from the colorimetric assay with the formula

$$\frac{OD_{a}}{OS_{ps}} \times \mu\text{mole}_{p} \times \frac{25}{0\ 1} \times \frac{1}{\mu\text{mole Hypro}} \times 100 = R\ \%$$

where OD is absorbance of pyrrole from the sample OD_p is absorbance of a standard solution of pyrrole, $\mu mole_p$ is the amount of pyrrole standard (usually 0 04 μmoles), 25/0 1 is the correction for the aliquot of toluene effluent taken for colorimetric assay and μmole Hypro is the amount of carrier hydroxyproline used in the assay (15 2 μmoles)

Accuracy and specificity

Prockop and Udenfriend (1960) demonstrated that the concentration of the chloramine-T is critical to the reaction Insufficient amounts of oxidant result in low yields of hydroxyproline and excessive amounts reduce the recovery, probably because of polymer formation The amount of compounds which consume chloramine T varies widely in biological

Table 1 Effect of varying amounts of chloramine T and hydrolyzate of embryonic homogenate on the assay of labeled hydroxyproline

Sample	Hypro C added dpm × 10⁻⁴	Chloramine T µmol s	Recovery as pyrrole %	Assayed Hypro ¹⁴C dpm × 10⁻⁴	Hypro C % of added dpm
Water	10.5	0.2	61.3	10.6	101
	10.5	0.3	58.6	10.4	99
	10.5	0.4	5[illegible].7	10.1	96
	10.5	0.6	4[illegible].4	10.2	97
Hydrolyzate					
6.0 ml	3.80	0.2	[illegible].2	3.69	9[illegible]
[illegible].0 ml	3.80	0.2	30.[illegible]	3.4	90
1.[illegible] ml	3.80	0.2	4[illegible].5	3.87	102
0.8 ml	3.80	0.2	49.9	3.59	9[illegible]

The hydrolyzate consisted of a homogenate from 10 day old chick embryos which was centrifuged at 100,000 × *g* for 1 h. The supernate was dialyzed, hydrolyzed, evaporated to dryness and dissolved in water. The amino acid content of the solution was about 100 mg/ml assayed by the method of Rosen (1957, 1963).

samples. Therefore, a number of assays with varying amounts of oxidant should be performed on each sample in order to establish optimal concentration.

As shown in Table 1, values obtained in pure samples were fairly constant with a threefold variation in the amount of oxidant. The % recovery of hydroxyproline as pyrrole decreased when interfering material was added in varying amounts but the assayed values for the hydroxyproline ^{14}C were not greatly affected even if the sample contained about 600 mg of ninhydrin positive material. With samples containing less than 50 mg of amino acids the calculated recoveries from 45 to 65 % in more than 1000 assays performed during this work. Within groups of similar samples assayed simultaneously the calculated recoveries varied by less than ± 3 %. The results of assays where the recovery of hydroxyproline as pyrrole was less than 40 % were rejected and the sample was purified before repeating the assay.

As earlier indicated, large excess of labeled proline caused erroneously high hydroxyproline values. By employing silicic acid chromatography a good separation of hydroxyproline from oxidation products of proline was achieved. Interference of labeled proline and other labeled compounds in final eluate is demonstrated in Table 2. When labeled prolines were used the observed radioactivity was not significantly higher than the background

Table 2 Interference from labeled compounds in final toluene eluate

Sample	dpm × 10^-3	Observed counts[a] cpm	% of added radioactivity
L-proline-14C	79.0	31[b]	< 0.1
L-proline-3,4-3H	130	30[c]	< 0.1
Algal protein hydrolyzate-14C	4700	14 400	0.7

[a] Observed cpm are not corrected for the background

[b] Mean of 70 assays range 25—37 cpm

[c] Mean of 30 assays range 6—43 cpm

The samples contained 1 mg of proline and 2 mg of hydroxyproline. Labeled compounds were added as indicated

in the counting system employed. A control containing these amounts of radioactive proline was run together with each series of samples. In a series of 153 separate assays this control of proline contamination gave two times a value higher than 0.1 %. The results of these two series were rejected. When the algal protein hydrolyzate-^{14}C was used as a sample a higher radioactivity was observed in the final toluene extract than could be accounted for by the proline-^{14}C content in the sample. Therefore when algal protein hydrolyzate-^{14}C was employed as a precursor the radioactivity of hydroxyproline was not assayed.

In order to minimize the possible interference from labeled proline the aliquots of samples for hydroxyproline assays were selected generally to contain less than 80 000 dpm of ^{14}C or 150 000 dpm of ^{3}H.

Statistical Methods

Curve fitting technique was employed for the calculation of the difference between the rates of total proline incorporation and hydroxyproline synthesis for the incorporation rate of sulphate as well as for the calculation of the significance between total proline incorporation and hydroxyproline synthesis in the tibiae incubated with varying concentrations of puromycin.

The problem was solved with linear regression analysis. General linear regression equation can be written

$$(1) \qquad y_i = \sum_{j=1}^{p} \beta_j x_{ij} + \varepsilon_i \quad (i = 1, 2, \ldots, n)$$

where y is the variable to be explained, x_j $(j = 1,2 \ldots p)$ are explanatory variables, and ε_i is the random disturbance term. The random disturbance terms are assumed to be independent and normally distributed with expectations 0 and variances σ^2. The coefficient vector β was estimated by applying the least square method

(2) $$\hat{b} = (x'x)^{-1} x'y$$

where b is the column vector of the least square estimates and $x'x$ is the covariance matrix of explanatory variables. The inverse of covariance matrix will be denoted by $(c^{jk})^{p \cdot p}$.

The confidence limits of regression estimate Y_i can be presented

(3) $$Y_i \pm t_p s \sqrt{\sum_{j=1}^{p} \sum_{k=1}^{p} c^{jk} x_{ij} x_{ik}}$$

where $$Y_i = \sum_{j=1}^{p} b_j x_{ij}$$

Let $$Q_0 = \sum_{i=1} (y_i - Y_i)$$ be the residual sum of squares and

$$Q_1 = \sum_{i=1}^{n} (Y_i - \bar{y})^2$$

the explained sum of squares. The total sum of squares

$$Q = \sum_{i=1} (y_i - \bar{y})$$

can be partitionate to independent components Q_0 and Q_1 according to indentity

(4) $$Q = Q_0 + Q_1$$

Residual variance s_0 can be estimated as $s_0^2 = \sqrt{\frac{Q_0}{n-p}}$ and multiple correlation $R = \sqrt{\frac{Q_1}{Q}}$. Regression model explains 100R % of the total sum of squares of variation in y.

In curvilinear relationship the original variables were transformed to $y' = f(y)$ and $x'_j = g(x_j)$ $(j = 1,2 \ldots p)$ for reducing the estimation process of regression function to general linear regression model. The final solution was the inverse transformation $y = y(y')$. Some initial conditions were taken in consideration: (1) in the case of total proline incorporation

and hydroxyproline synthesis as x (0) = 0 (2) x (0) = 100 in the case of puromycin effect The statistical analysis was performed with an IBM 1130 computer in the Department of Applied Mathematics University of Turku by Mr Heikki Lopponen M Sc

List of special reagents

Isotopes Uniformly labeled L-proline C 180—200 μCi/μmole L proline 3 4 ³H 5000 μCi/μmole sodium sulphate ³⁵S 168 μCi/μmole uridine 2 C, 30 μCi/μmole, orotic acid 6 C, 6 3 μCi/μmole and algal protein hydrolyzate C, 1390 μCi/mg were obtained from New England Nuclear Corp, Boston, Mass U S A The algal protein hydrolyzate contained 6 % of L proline C and 10 % of the total C content was accounted for by amino acid Uniformly labeled toluene C and n hexadecane 1 ³H were obtained from The Radiochemical Centre Amersham England The activity of these compounds was assayed by comparison to tritiated water and benzoic acid C standards which were obtained from the U S National Bureau of standards Hydroxyproline C 4 μCi/μmole was from a batch prepared biosynthetically by Katz Prockop and Udenfriend (196)

Antibiotics Antibiotics were obtained from following companies chloramphenicol Merck Sharp and Dohme, Rahway N J U S A penicillin G American Quinidine Co New York N Y, U S A, puromycin hydrochloride Nutritional Biochemicals Co Cleveland Ohio U S A, streptomycin sulphate Chas Pfizer & Co New York, N Y, U S A

Nucleotides ATP disodium salt Sigma grade NAD, disodium salt, grade III β NADH disodium salt grade III NADPH tetrasodium salt type I sRNA, type III from yeast were purchased from Sigma Chemical Co St Louis Mo U S A

Metal ion chelators Cupron (α benzoin oxime) ACS reagent diethyldithiocarbamic acid sodium salt highest purity α α-dipyridyl ACS reagent o phenanthroline ACS reagent Tiron® (4 5 dihydroxy m benzenedisulfonic acid disodium salt) ACS reagent were purchased from Fisher Scientific Co King of Prussia Pa U S.A

Miscellaneous reagents Ascorbic acid U S A purity creatine phosphate disodium salt hydrate Sigma grade glutathione grade III DL-α lipoic acid reduced form were purchased from Sigma Chemical Co α ketoglutaric acid puriss from Fluka A G Buchs Switzerland 2 amino 4 hydroxy 6 7 dimethylpteridine highest purity from Aldrich Chemical Co Milwaukee Wis U S A Eagle s minimum essential medium with Earle s balanced salt solution from Microbiological Associates Inc, Bethesda Md U.S A platinum oxide catalytic reagent grade from J Bishop & Co Malvern Pa U S A

RESULTS

I SUPPORTING EVIDENCE FOR THE HYDROXYLATION OF PROLINE IN A PEPTIDE PRECURSOR OF COLLAGEN

General biosynthetic characteristics of tibiae *in vitro*

Incorporation of proline and synthesis of hydroxyproline Considerable change in the rates of proline incorporation and hydroxyproline synthesis was observed Fig 1 shows that the total incorporation of tritiated proline increased with incubation periods up to 21 h The rate of incorporation however appeared to decrease after an incubation period of 6 h The synthesis of labeled hydroxyproline paralleled total incorporation up to 6 h thereafter a decrease was observed

Change in the rates of proline incorporation and hydroxyproline synthesis was more apparent when shorter labeling periods were employed As shown in Fig 2 A and 2 B the rates of proline incorporation and of hydroxyproline synthesis increased 2 to 3 fold between 0 and 6 h and decreased thereafter At around 8th h the rate of hydroxyproline synthesis was significantly ($P < 0\ 05$) lower compared to that of proline incorpora tion which remained relatively high (Fig 3) Similar results were obtained when experiments described in Fig 2 were repeated employing Eagle s medium (Eagle 1959)

When L proline ^{14}C was used as a label 21—25 % of the incorporated ^{14}C was consistently recovered as hydroxyproline ^{14}C When L proline 3 4 ^{3}H was employed as a precursor the hydroxyproline ^{3}H content varied 6—24 % apparently depending on the batch of the isotope used Consistent results however were obtained when a single preparation was used for a series of experiments

Hydrolyzates of samples in which ^{14}C proline was employed as a precursor were chromatographed on paper (Whatman No 1) using phenol 1 % (v/v) ammonia and *n* butanol acetic acid water (5 1 4) as solvent systems Amino acids were located with ninhydrin (Sigma Chemical Co)

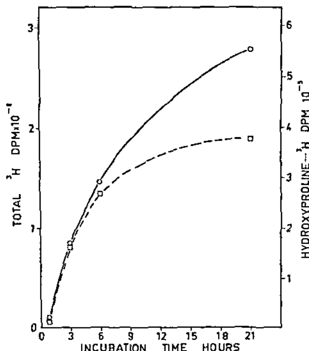


Fig 1 Cumulative incorporation of L proline 3 4 ³H into protein and synthesis of hydroxyproline ³H in embryonic cartilage

Each sample contained one tibia from a 9 day old embryo and it was incubated with 20 μCi of L-proline 3 4 ³H for the periods of 1 3 6 or 21 h Total ³H incorporated ○ hydroxyproline ³H synthesized □

The distribution of radioactivity on chromatograms was assayed with windowless strip counter (Packard Instrument Co) Over 95 % of the radioactivity on chromatograms was recovered as proline ¹⁴C and hydroxyproline ¹⁴C

Incorporation of uridine 2 ¹⁴C In order to estimate the rate of ribonucleic acid synthesis tibiae were incubated with uridine 2 ¹⁴C The radioactivity in samples was assayed with the same technique as described for the total incorporation of labeled proline As shown in Table 3 the rate of uridine incorporation decreased rapidly *in contrast to the concurrent increasing* rate observed in proline incorporation (Fig 2) Similar results were obtained when orotic acid 6-¹⁴C was employed as a precursor (not shown)

Incorporation of sulphate ³⁵S Incorporation of radioactive sulphate has been used as a measure for the synthesis of sulphated acid mucopolysaccharides Radioactive sulphate in tibiae was assayed with the same

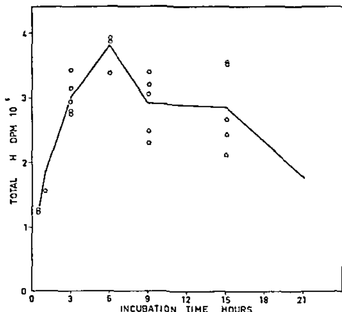


Fig 2 A Effect of incubation interval on the rate of total L proline 3 4 H incorporation into protein in embryonic cartilage

Each sample contained two tibiae from a 10 day old chick embryo The samples were incubated at 37 for the period indicated Twenty μCi of L proline 3 4 H were added in the beginning of the last 30 min of each incubation period The curve represents the mean of the incorporation rate at each point of time tested Total H incorporated dpm O

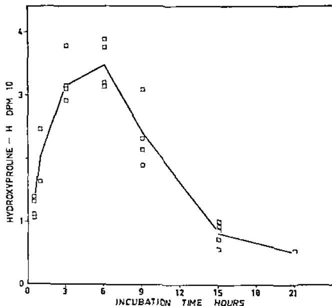


Fig 2 B Effect of incubation interval on the rate of hydroxyproline H synthesis in embryonic cartilage

The samples are the same as in Fig 2 A. Hydroxyproline H synthesized dpm □

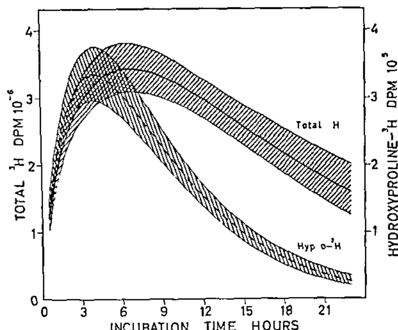


Fig 3 Statistical comparation of the rates of total proline incorporation and synthesis of hydroxyproline in embryonic cartilage

The values are from Fig 2 A and 2 B The equation for the curve of total incorporation was calculated $y = 93\,442\ t^{\ldots}\ e^{-0\ldots}$ where y is the rate of incorporation in dpm/30 min and t is the incubation time in h

The model explains 83 57 % (100 R) of the change in the rate of proline incorporation multiple correlation R = 0 914 According to F test this model is statistically significant $F(2\ ?7) = 68\ 65$ $P < 0\ 001$

The equation for the curve of hydroxyproline synthesis was calculated $y = 24\ 52\ t^{\ldots}\ e^{-0\ldots}$ where y is the rate of hydroxyproline synthesis in dpm/30 min and t is the incubation time in h 100P = 89 64 % r = 0 947 F(?8) = 121 15 $P < 0\ 001$ Solid line total H dotted line hydroxyproline H The shadowed areas represent 95 % confidence intervals of the regression estimates

Table 3 Effect of incubation interval on the rate of uridine 2 ¹⁴C incorporation into tibiae

Preincubation period h	Total C dpm × 10⁻⁴
0	2 32
0	. 11
3	1 16
5	0 48

Four tibiae from 10 day old embryos were incubated with 5 µCi of uridine-2 ¹⁴C for 1 h either immediately after the dissection (indicated by zero) or after the preincubation period of 3 or 5 h.

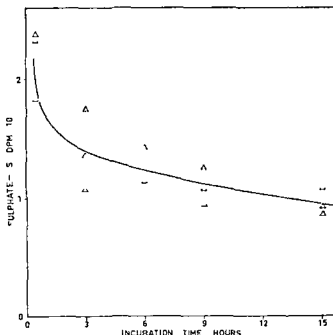


Fig 4 Effect of incubation interval of the rate of sulphate S incorporation into embryonic cartilage

Each sample contained 2 tibiae from 10 day old chick embryos and 30 μCi of sodium sulphate ^{35}S were added together with 20 μCi of L proline 3 4 H for the last 30 min of each incubation period The equation of the curve was calculated from the mean of the incorporation rates at each point $y = 125\,73 - 2\,46t + 89\,18/(t+1)$ where y is the rate for sulphate incorporation in dpm/30 min and t is the time of incubation in h 100F = 99 61 % R = 0 998 F(2 9) = 257 61 P < 0 005 (Values for the rate of proline incorporation and hydroxyproline formation are included in Fig 2 A and 2 B)

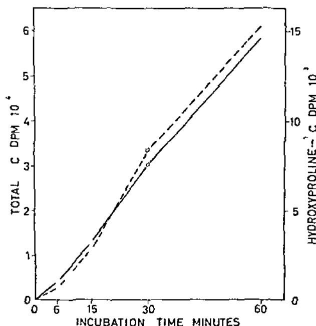
technique as described for total proline Fig 4 shows that the rate of sulphate incorporation was at its highest immediately after the dissection of tibiae

Incorporation of radioactive proline and synthesis of hydroxyproline in subcellular fractions Tibiae were incubated with L proline ^{14}C for periods indicated in Table 4 After the incubation the samples were cooled in ice bath and homogenized in 9 ml of buffered sucrose solution (Bloemendal Bont and Benedetti 1964) The homogenate was divided in three fractions by successive centrifugations at 15 000×*g* for 20 min and at 100 000×*g* at 4° for 1 h in a Spinco L ultracentrifuge (Beckman Instruments Inc Fullerton Calif, U S A) The supernate from the second centrifugation is referred to as soluble fraction All fractions were dialyzed exhaustively with 100 mg of L proline against distilled water at 4° Total radioactivity and hydroxyproline ^{14}C content in the retentates were assayed

Table 4 Accumulation and distribution of total C and hydroxyproline C in subcel lular fractions of embryonic cartilage

Incul ation time min	15,000×g sediment			100 000×g sediment			Soluble fractio	
	Total C dpm×10⁻⁴	Hypro C		Total C dpm×10⁻⁴	Hypro C		Total C dpm×10⁻⁴	Hypr
		dpm×10⁻²	% of total ¹C		dpm×10	% of total C		dpm×10
6	2 50	‿60	22	0 88	2 20	ɔ	4 91	0 54
15	8 50	16 1	19	1 79	3 8?	21	11 6	1 °4
30	14 8	—	—	—	—	—	3‗ 5	2 61
60	39 3	—	—	8 2ɔ	14 8	18	—	—
1 0	107	‗31	22	21 6	46 0	21	149	17 9

Each sample contained 6 tibiae from 10 day old embryos After a preincul ation perio l of 10 min at 37 , 10 µCi of L proline C were added and the incubation was continued for the perio ls indicated The samples were homogenized and fractionate l in three fractions by successive centrifugations at 15 000×g for °0 min and 100 000×g for 1 h at 4 (See details in the text)



F g 5 1 cumulation of proline ¹⁴C and of hydroxyproline ¹ C in the 100,000×g s diment of embryonic artilage homogenate

Fxperim ntal cond tion were th same as in Tal le 4 Total C O hy lroxyp roline C □

A progressive increase in total ^{14}C and hydroxyproline ^{14}C was observed in all fractions (Table 4). The hydroxyproline ^{14}C content, however, remained constant. Fig. 5 demonstrates the progressive increase of total ^{14}C and of hydroxyproline ^{14}C in the $100\,000 \times g$ sediment, in a similar experiment as that shown in Table 4. Similar results were also obtained in three successive experiments.

The relative distribution of radioactivity among the fractions appeared to depend primarily on the efficiency of the homogenization. When tibiae were homogenized at 4500 rev/min for 2 min, the incorporated radioactivity was distributed approximately equally among the three fractions, but when the tissue was homogenized at 1500 rev/min, about 80 % of the label was recovered in $15\,000 \times g$ sediment. Washing the particulate fractions did not markedly change the distribution patterns of total radioactivity or hydroxyproline content among the fractions. Distribution of hydroxyproline ^{14}C or ^{3}H paralleled that of total ^{14}C or ^{3}H incorporated. In the soluble fraction, however, the content of hydroxyproline ^{14}C was consistently lower than that in the other two fractions.

None of the experiments indicated any definite lag period between the total incorporation of proline and the appearance of hydroxyproline in the fractions studied. No definitive conclusion can be drawn from the precursor product relationship between $100\,000 \times g$ sediment and either of the other two fractions.

Effect of Anaerobic Conditions on the Incorporation of Proline and Synthesis of Hydroxyproline

The effect of different oxygen tensions on the rate of total proline incorporation and hydroxyproline formation was studied. Two tibiae from 10 day old embryos were placed in Warburg flask containing 3 ml of incubation medium. The medium was saturated with gas mixtures by bubbling it vigorously at room temperature for 15 min. Two μCi of L proline ^{14}C in 0.2 ml of medium were pipetted into the sidearm of the flask. The flasks were flushed with gas mixtures for 20 min, closed tightly with rubber stopper and incubated at 37° for 10 min. The radioactivity was tipped in the main chamber and incubation was continued for 1 h. The flasks were cooled in ice bath and the tibiae were processed as described (p. 15).

As shown in experiment 1 (Table 5) oxygen content did not affect the hydroxylation of incorporated proline. In samples incubated in 100 % nitrogen atmosphere the total ^{14}C incorporated was about the same as in matched control samples incubated in aerobic conditions (experiments

Table 5 Effect of various oxygen concentrations on the incorporation of labeled proline and synthesis of hydroxyproline by embryonic cartilage

Oxygen content volume %	Total C dpm×10⁻⁴	Hypro C	
		dpm×10	% of total C
Expt 1			
0	6 87	1 44	2 1
5	4 38	9 ,2	2[illegible]
10	5 82	13 2	23
15	4 68	10 8	[illegible]3
21	9 2[illegible]	20 1	22
50	3 88	8 31	[illegible]1
95	4 53	8 89	20
Expt 2			
0	17 3	2 80	1 6
95	16 8	34 1	20
0	13 8	1 60	1 2
95	15 3	33 5	[illegible]
0	14 3	[illegible] 90	2 0
95	1[illegible] 9	29 8	23
Expt 3			
0	8 40	1 81	[illegible]
95	10 2	21 3	21
0	7 30	1 42	1 9
95	11 5	2 5	20

in air

Two tibiae from 10 day old embryos were incubated in Warburg fla ks containing 3 ml of Krebs 2 A medium and 2 μCi of L proline C The gas mixtures contained oxygen as indicated 5 % carbon dioxide and nitrogen Zero indicates that the samples were incubated in 100 % nitrogen atmosphere In experiments 2 and 3 matched pairs of tibiae were employed (See details in the text)

2 and 3) whereas the formation of hydroxyproline decreased to about one tenth in samples incubated under nitrogen In experiment 3 a slight decrease in total incorporation of radioactivity was observed As a general conclusion the hydroxylation of proline is more sensitive to decreased oxygen tension than the peptide synthesis

Effect of αα Dipyridyl on the Synthesis of Collagen

The effect of αα dipyridyl a chelator for ferrous iron, on the incorporation of proline and the synthesis of hydroxyproline was tested

Table 6 Effect of α α dipyridyl on the incorporation of L proline 3 4 H and on the formation of hydroxyproline ³H

Sample	α α dipyridyl M	Total H		Hypro H	
		dpm × 10	% of control	dpm × 10	% of total H
Expt 1					
Control		8 33		13 5	16
Test	10^{-3}	0 68	8 2	—	—
Control		6 14		9 90	16
Test	10	5 70	93	0 68	1[illegible]
Control		6 96		8 [illegible]0	13
Test	10^{-4}	8 1[illegible]	11[illegible]	0 [illegible]3	0 9
Expt 2					
Control		10 3		11 7	11
Test	10^{-3}	1 [illegible]0	1[illegible]	—	—
Control		8 4[illegible]		10 3	1[illegible]
Test	10	8 54	101	0 43	0 5
Control		9 34		11 3	12
Test	10^{-4}	9 12	98	0 73	0 8

Matched pairs of tibiae from 10 day old embryos were incubated in the presence of various concentration of α α dipyridyl After a preincubation period of 30 min 20 μCi of L proline 3 4 H were added and the incubation was continued for an additional period of 60 min at 37

As shown in Table 6, α,α′ dipyridyl in concentrations of 1×10^{-3}M or 1×10^{-4}M prevented the hydroxylation of proline without affecting the total proline incorporation The results suggested that ferrous iron is essential for the hydroxylation of proline

Effects of Puromycin and Chloramphenicol on the Incorporation of Proline and Sulphate and on the Synthesis of Hydroxyproline

Puromycin Puromycin was found to be a potent inhibitor for the protein synthesis in embryonic cartilage The formation of hydroxyproline appeared to be more sensitive to puromycin than the synthesis of other proteins Fig 6 shows that both the total isotope incorporation and the formation of labeled hydroxyproline were inhibited to about the same degree with up to puromycin concentration of 5 μg/ml With higher concentrations however the synthesis of hydroxyproline was consistently

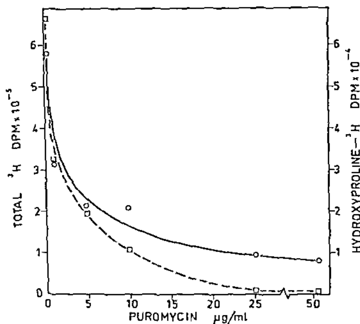


Fig 6 Effect of puromycin on the incorporation of labeled proline and the synthesis of hydroxyproline by embryonic cartilage

One tibia from 9 day old chick embryo was incubated for 30 min with various concentrations of puromycin and then 20 μCi of L proline 3 4 H were added for an additional incubation period of 60 min Total ³H O hydroxyproline H □

inhibited to a greater extent than the incorporation of proline Table 7 is a summary of the dose effect of puromycin on the incorporation of proline and sulphate as well as on the synthesis of hydroxyproline The incubation periods varied from 0 5 to 6 h, which apparently did not affect the degree of inhibition observed The inhibition in sulphate ³⁵S incorporation was about the same as that in total proline incorporation but it was considerably less than the inhibition in hydroxyproline synthesis Based on the values in Table 7 dose response curves were calculated for total incorporation hydroxyproline synthesis and sulphate incorporation Fig 7 shows that the difference between proline and hydroxyproline was significant ($P < 0\ 05$), when the concentration of puromycin was about 7 μg/ml or higher The curve of sulphate incorporation paralleled that of proline (not shown)

The effect of puromycin was further studied by comparing simultaneous incorporation of proline ³H and of a ¹⁴C labeled amino acid mixture in tibiae (Table 8) No difference between incorporation rates of proline

Table 7 Effect of puromycin on the incorporation of proline and sulphate and on the synthesis of hydroxyproline

Puromycin µg/ml	Total ³H or ¹⁴C % of control			Hypro ³H or ¹⁴C % of control			Sulphate ³⁵S % of control		
0 0[illegible]	96			99			98		
0 2	98			100			100		
1 0	70			64			—		
2 0	60	50	41	54	43	42	44	42	45
5 0	36			36				—	
8 0	5[illegible]	42	31	[illegible]6	32	14		—	
10	34			13				—	
20	23	2[illegible]	17	[illegible]	6	6	23	21	20
25	17			2				—	
40	20	17	12	2	3	2		—	
50	12	6		1 5	0 [illegible]			—	

One or two tibiae from 10 day old embryos were incubated in the presence of puromycin as indicated After an incubation period of 20 min 2 µCi of L proline ¹⁴C or 20 µCi of L-proline-3,4 ³H were added and the incubation was continued for periods of ½—6 h Into the samples for which sulphate values are quoted, 18 µCi of sodium sulphate ³⁵S were added together with tritiated proline

and the mixture of labeled amino acids was observed as indicated by the ratio of ^{3}H to ^{14}C This remained constant up to a puromycin concentration of 50 µg/ml when the observed inhibition in total proline incorporation was 90 % The result indicated that proteins or peptides synthesized in the inhibited samples had an amino acid composition similar to those synthesized in the control samples

The reversion of the puromycin effect As shown in Table 9 (Experiments 1 and 2) during the first 3 h incubation with puromycin a marked inhibition both in total incorporation and in hydroxyproline formation was observed At 20 µg/ml dose level of puromycin the inhibition in hydroxyproline synthesis was disproportionally greater than that in total proline incorporation During the following incubation without puromycin the inhibition was abolished especially in hydroxyproline formation The reversion however was not complete (Experiments 3 and 4)

Chloramphenicol Relatively high concentration of chloramphenicol was necessary to observe any effect No difference between the total incorporation of proline and hydroxyproline synthesis was observed The incorporation of sulphate paralleled that of proline (Table 10)

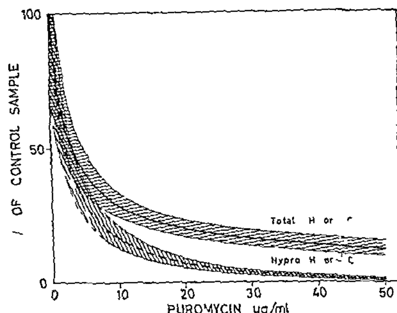


Fig 7 Statistical analysis on the disproportional effect of puromycin on total incorporation of labelled proline and on synthesis of hydroxyproline

The curves were calculated from the values presented in Table 7 The equation for puromycin effect on total proline incorporation was calculated y = 99 083 (1 + tgh [c]) [1/(1 + [c])] where y is % of the radioactivity in the control sample and [c] is the concentration of puromycin. R = 0 9.7, 100R = 87 72 %, F(2 16) = 57 16, P < 0 001

The equation for inhibition in hydroxyproline was calculated y = 93 44 e [1/(1 + [c])] where y is % of the radioactivity in the control sample and [c] is the concentration of puromycin 100P = 95 41 % R = 0 97, F(2 17) = 178 524 P < 0 001 The shadowed areas represent 95 % confidence intervals of the regression estimates

Table 8 Effect of puromycin on simultaneous incorporation of L proline 3 4 H and ¹⁴C labeled mixture of amino acids

Puromycin µg/ml	Total H		Total C		³H/¹⁴C ×100
	dpm×10⁻⁴	% of control	dpm×10	% of control	
Control	8 43		4 95		1‹ 1
5 0	3 46	41	2 31	47	15 0
Control	7 88		5 06		15 6
25	1 48	19	0 89	18	16 6
Control	6 34		3 82		1f 6
50	0 64	10	0 38	10	16 8

One tibia from a 10-day old embryo was incubated with various concentrations of puromycin as indicated and the other tibia from the same embryo served as a control After a preincubation period of 20 min 20 µCi of L proline 3,4-³H were added together with 5 µCi of algal protein hydrolyzate C and the incubation was continued for another 30 min

Table 9 Reversion of the effect of puromycin

Puromycin µg/ml	Incubation time h	Total ^{3}H		Hypro ^{3}H	
		dpm×10^{-5}	% of control	dpm×10^{-4}	% of control
Expt no					
1 Control	3	8 83		12 6	
2 0	3	3 64	41	5 .9	4?
2 Control	3	10 0		12 4	
20	3	2 ?,	2.	0 72	.8
3 Control	3 + ½ + 3	14 3		11 0	
2 0	3 + ½ + 3	8 69	61	8 40	76
4 Control	3 + ½ + 3	11 6		10 0	
20	3 + ½ + 3	80	6,	5 04	50

* Incubation with puromycin

One tibia from 9 day old chick embryo was incubated with 2 0 or 20 µg/ml of puromycin and the other tibia from the same embryo served as a control In the experiments 1 and 2 20 µCi of L proline 3 4 H were added in the beginning of incubation In the exp riments 3 and 4 the test samples were fir t incubated with antibiotic for 3 h Thereafter, the tibiae were transferred for 30 min to the second tubes containing Krebs 2 \ medium transferred to the third tubes and then 20 µCi of tritiated proline were added to all samples for an additional incubation period of 3 h at 3"

Table 10 Effect of chloramphenicol on the incorporation of L proline 3 4 H and sodium sulphate S and on the synthesis of hydroxyproline H

Chloram phenicol mg/ml	Total ^{3}H		Hypro ^{3}H		Sulphate ^{35}S	
	dpm×10^{-4}	% of control	dpm×10	% of control	dpm×10	% of control
Control	1 80		2 ,9		7 60	
0 0.	1 8	104	? ,7	100	9 10	1?0
Control	1 8		3 12		56	
0 5	1 60	90	2 14	69	5 44	72
Control	1 61		2 6.		6 64	
1 5	0 81	50	1 2.	47	3 96	60
Control	1 48		1 ,3		6 92	
? 5	0 10	6 8	0 14	8 1	0 78	11

Two tibiae from 10 day old chick embryos were incubated with varying concentrations of chloramphenicol at 3 for 20 min Thereafter 0 µCi of L proline .,4 ^{3}H and 18 µCi of sodium sulphate ^{35}S were added into the tubes and incubated for 30 min

Effects of puromycin and chloramphenicol on the distribution of radioactivity between subcellular fractions Juva and Prockop (1964) reported that labeled polypeptides accumulated in the 100 000×g supernate of chick embryo tibia homogenate when protein synthesis was inhibited with puromycin The hydroxyproline content in the soluble fraction was about one tenth of that observed in particulate fractions when the overall inhibition in total proline incorporation was about 90 % This observation was confirmed and a comparison was made to the effect of chloramphenicol (Tables 11 and 12) As shown in Tables 11 and 12 labeled polypeptides accumulated in soluble fraction of the tissue homogenate when protein synthesis was inhibited with puromycin This fraction contained less hydroxyproline than the polypeptides recovered from the other two fractions Although comparable inhibitions in protein synthesis were obtained with both antibiotics no increase was observed in the relative amount of the non dialyzable radioactivity recovered in the soluble fraction of chloramphenicol treated samples A slight increase in radioactivity in the 100 000×g sediment was observed (Table 12 experiments 3 and 4) but this effect of chloramphenicol was not further investigated Chloramphenicol did not affect the content of labeled hydroxyproline in any of the three fractions

The results were interpreted so that puromycin interrupts the synthesis of a proline rich polypeptide precursor of collagen When large con

Table 11 Effect of puromycin and chloramphenicol on the distribution of total C and hydroxyproline C in subcellular fractions

Concentration of antibiotics	15 000×g sediment			100 000×g sediment			Soluble fracti[on]	
	Total C dpm×10	Hypro C		Total C dpm×10^{-4}	Hypro C		Total C dpm×10^{-4}	Hypro
		dpm×10	% of total C		dpm×10	% of total C		dpm×10^{-3}
Control	51 6	1 8	25	36 9	108	29	60 7	69 8
PM 40 µg/ml	2 [illegible]3	1 08	4 0	1 10	0 53	4 8	5 19	0 41
CA 1 25 mg/ml	25 6	64 0	[illegible]5	2[illegible] 4	65 9	29	16 9	20 0
CA 2 50 mg/ml	2 5[illegible]	5 24	21	17 6	3 96	23	14 2	1 72

PM = puromycin CA = chloramphenicol

Two tibiae from 10 day old embryos were incubated with puromycin or chloramphenicol After incubation at 37 for 20 min 20 µCi of L proline C were added for an additional incubation period of 30 min The samples were treated as indicated in Table 4

Table 1. Effect of puromycin and chloramphenicol on the distribution of total ^{14}C or ^{3}H and hydroxyproline ^{14}C or ^{3}H among homogenate fractions of tibiae

Concentration of antibiotics	^{14}C or ^{3}H in fraction / Total ^{14}C or ^{3}H in samples × 100			Hypro ^{14}C or ^{3}H in fraction / Total ^{14}C or ^{3}H in fraction × 100		
	15 000×g sediment	100 000×g sediment	Soluble fraction	15 000×g sediment	100 000×g sediment	Soluble fraction
Expt no	%	%	%	%	%	%
1 Control	36	36	28	23	25	19
PM 8 0 μg/ml	22	13	65	16	19	9 0
PM 40 μg/ml	22	12	66	4 1	4 6	0 7
2 Control	41	36	23	12	13	10
PM 8 0 μg/ml	47	25	28	11	7 6	4 8
PM 40 μg/ml	35	20	45	4 2	2 8	0 8
3 Control	47	23	30	25	29	[illegible]
PM 40 μg/ml	30	12	58	4 0	4 8	0 8
CA 1 25 mg/ml	40	34	6	25	[illegible]	12
CA 2 50 mg/ml	44	31	25	21	23	12
4 Control	47	24	29	6 6	9 6	7 2
CA 2 0 mg/ml	41	36	23	8 0	9 6	4 1
5 Control	75	14	11	6 5	6 8	5 4
PM 40 μg/ml	30	20	50	—	—	—
Control	74	12	14	6 6	5 7	4 4
CA 2 5 mg/ml	76	12	12	1 8	4 6	2 5

PM=puromycin CA=chloramphenicol

The experimental conditions were the same as in Table 11, except that in the experiments 1 and 3 2 0 μCi L proline ^{14}C were used as a labeled precursor

centrations of puromycin are present the polypeptides which continue to be synthesized are of insufficient size to serve as substrate for the hydroxylation

II HYDROXYLATION OF PROLINE IN A PEPTIDE PRECURSOR OF COLLAGEN

The results about the inhibitory effect of puromycin suggested that hydroxylation of proline occurs in a peptide precursor of collagen The protein synthesis continued in embryonic cartilage under nitrogen when the synthesis of hydroxyproline was inhibited These observations pointed

at a possibility of finding a peptide precursor of collagen This concept was further supported by the effect of $\alpha\alpha'$ dipyridyl which inhibited the synthesis of hydroxyproline without affecting the incorporation of proline

The precursor was first demonstrated in a particulate form and later it was obtained in a solubilized form The enzymatic system required for the hydroxylation of peptide bound proline was partially characterized

Hydroxylation of Proline in Particulate Fractions

Preparation and incubation of particulate fractions Twelve to 50 tibiae from 10 day old chick embryos were incubated under nitrogen with 40—300 μCi of L proline ^{14}C at 37° for 1 h Thereafter the tibiae were homogenized

Table 15 Synthesis of hydroxyproline C in particulate fractions of embryonic cartilage

Labeled precursor	Temperature C	Total C	Hypro C	
		dpm×10⁻⁴	dpm×10	% of total C
1 00×g sediment	4	10 2	3 90	3 8
	37	7 05	3 90	4 8
	4	5 50	65	5 1
	37	6 85	5 30	7 7
15 000×g sediment	4	3 45	0 80	2 3
	37	4 50	1 50	3 3
	4	1 86	0 95	5 1
	37	1 61	1 40	8 7
	4	3 09	3 10	1 0
	37	4 00	6 05	1 5
100 000×g sediment	4	8 30	0 80	0 9[illegible]
	3	6 90	2 60	3 8
	4	3 64	1 95	5 4
	3[illegible]	3 15	2 45	[illegible]8

Particulate fractions of tibia homogenate labeled with L proline C under nitrogen were added to the hydroxylating system which consisted of 0 25 M sucrose 0 05 M tris buffer pH 6 0 004 M $MgCl$ 0 0 M KCl 0 02 M creatine phosphate 0 00 M ATP and 10 ml of 15 000×g supernate from 9 day old chick embryos The total volume of the samples was 20 ml The samples were incubated in air for 2 h and centrifuged at 100 000×g for 1 h at 4 The total radioactivity and hydroxyproline C content in the sediments were assayed

in the buffered sucrose solution as described earlier (p 28) The homogenate was centrifuged at 1200×g for 10 min and the supernate was centrifuged at 15,000×g for 15 min The supernate from the second centrifugation was divided into approximately equal portions and centrifuged at 100,000×g for 1 h Fractionation was carried out at 4° The sediments and supernate were stored at —20°

The particulate fractions were incubated in a hydroxylating system as described by Peterkofsky and Udenfriend (1963b) Embryonic super

Table 14 Synthesis of hydroxyproline 100 000×g sediments of prelabeled tibiae under various incubation conditions

Experimental conditions	Temperature C	Total C dpm×10⁻⁴	Hypro C	
			dpm×10	% of total C
Expt 1				
Complete system	4	͜ 17	2 60	5 0
	3͵	9 ͜9	15 9	1͵
minus creatine P and ATP	37	7 5͵	1° 0	16
with 100 000×g supernate of embryonic homogenate	3"	9 ͜5	15 3	1"
Expt 2				
Complete system	4	5 00	͜ 50	͜ 0
,	3	5 45	58 8	11
with boiled embryonic supernate	3	4 40	47 1	11
Expt 3				
Complete system	4	12 6	4 15	3 3
, ,	37	15 4	1͵ "	11
minus embryonic supernate	37	10 1	3 00	3 0
Complete system	4	3 62	1 40	3 9
Boiled embryonic supernate	37	͜ 10	6 90	14
Boiled pellet	37	3 34	1 57	4 7
Expt 4				
Complete system	4	4 80	2 3͜	4 9
	3͵	4 75	4 90	10
+EDTA 5×10⁻⁴M	37	5 42	2 45	4 5
+EDTA 1×10⁻⁴M+FeSO 1×10 M	37	4 60	4 65	10

The experimental conditions were the same as in Table 13 The total radioactivity and hydroxyproline C content in the samples were assayed in hot trichloroacetic acid extracts

nate was prepared from 9 day old chick embryos They were homogenized for 15 sec in 1/9 their volume of 2.5 M sucrose in a Sorvall Omni Mixer (type OM Ivan Sorvall Inc Norwalk Conn U S A) and the homogenate was centrifuged at 15 000×g for 15 min An increase in hydroxyproline ^{14}C was observed in all particulate fractions (Table 13) The hydroxyproline content in samples incubated at 4° varied because the particulate fractions were from a different batch of preparations The 100 000×g sediments were found to give most constant results as judged by the increase in labeled hydroxyproline

When free proline ^{14}C was employed as a substrate in the same system the relative yield of labeled hydroxyproline was considerably less than that observed when the prelabeled sediments were employed In six separate experiments 0 001—0 02 % of the added proline ^{14}C was recovered as hydroxyproline ^{14}C

The hydroxylation was not dependent on the addition of creatine phosphate and ATP (Table 14) The 100 000×g supernate of embryo homogenate was found equally active as a 15 000×g supernate from the same homogenate This fraction is referred to as embryonic supernate hereafter The hydroxylation was dependent on the soluble fraction of chick embryo homogenate Essential factor or factors contributed by embryonic supernate were not affected by boiling it for 10 min Boiling the pellet on the contrary, destroyed its property to serve as a precursor for hydroxyproline The hydroxylation was completely inhibited by EDTA but when equimolar amount of ferrous iron was added the activity was restored The heat stable factor contributed by the embryonic supernate was lost after dialysis Addition of ferrous iron did not restore its activity

The results indicated that particulate fractions contained both the substrate and the enzymatic component for the hydroxylation

Solubilization and Partial Characterization of the Peptide Precursor of Collagen

Various conditions for solubilizing substrate for *hydroxylation from* 100 000×g pellets of prelabeled tibiae homogenate were tested With dilute acid or neutral buffers only minimal amounts of bound proline were extractable About one third of the nonhydroxylated material was solubilized at pH 10 and about two thirds at pH 13 The substrate was also solubilized with 1 M NaCl and with 8 M urea None of these treatments reduced substrate activity of solubilized material The extraction with 1 M NaCl or 1 M KCl gave preparations which contained the substrate

in the buffered sucrose solution as described earlier (p 28) The homogenate was centrifuged at $1200 \times g$ for 10 min, and the supernate was centrifuged at $15{,}000 \times g$ for 15 min The supernate from the second centrifugation was divided into approximately equal portions and centrifuged at $100\,000 \times g$ for 1 h Fractionation was carried out at 4° The sediments and supernate were stored at —20°

The particulate fractions were incubated in a hydroxylating system as described by Peterlofsky and Udenfriend (1963*b*) Embryonic super

Table 14 Synthesis of hydroxyproline 100 000×g sediments of prelabeled tibiae under various incubation conditions

Experimental conditions	Temperature C	Total C dpm×10	Hypro C	
			dpm×10	% of total C
Expt 1				
Complete system	4	5 17	2 60	5 0
	37	9 59	15 9	17
minus creatine P and ATP	37	7 57	12 0	16
with 100 000×g supernate of embryonic homogenate	37	9 95	15 3	17
Expt 2				
Complete system	4	5 00	2 50	5 0
	37	5 45	58 8	11
with boiled embryonic supernat	37	4 40	47 1	11
Expt 3				
Complete system	4	12 6	4 15	3 3
	37	15 4	17 7	11
minus embryonic supernate	37	10 1	3 00	3 0
Complete system	4	3 62	1 40	3 9
Boiled embryonic supernate	37	5 10	6 90	14
Boiled pellet	37	3 34	1 57	4 7
Expt 4				
Complete system	4	4 80	2 35	4 9
	37	4 75	4 90	10
+EDTA 5×10 M	37	5 49	2 45	4 5
+EDTA 1×10 M+FeSO 1×10 M	37	4 60	4 65	10

The experimental conditions were the same as in Table 13 The total radioactivity and hydroxyproline C content in the samples were assayed in hot trichloroacetic acid extracts

Table 15 Hydroxylation of solubili ed substrate by embryonic supernate

Experimental con litions	Tempera ture C	Total ¹C dpm×10⁻³	Hypro ¹C dpm×10⁻³	Hypro ¹C % of total C
Expt 1				
Compl te sy tem minus FeSO	4	[illegible] 20	[illegible] 2	4 2
with FeSo 5×10⁻⁴M	37	6.30	3 2	5 1
with FeSo 5×10⁻⁴M	37	4 60	9 6	21
with FeCl 5×10⁻⁴M	37	4 80	10 4	22
Expt 2				
Complete ystem	4	3 2[illegible]	16 4	5 1
" "	37	3 [illegible]	70 5	[illegible]2
with boiled embryonic supernate	37	3 09	21 6	7 0
Expt 3				
Complete system	4	5 2[illegible]	2 8	[illegible] 3
" "	37	5 80	9 2	16
with boiled substrate	37	5 1[illegible]	8 2	16
Expt 4				
Complete system	4	[illegible] 20	2 7	5 2
" "	37	[illegible] 10	10 7	21
with dialyzed embryonic supernate	37	5 05	2 5	5 0
with dialyzed embryonic supernate + dialyzate	37	5 10	4 3	8 4

Complete system contained dialyzed 1 M KCl extract from 100 000×g ediment of anaerobically prelabeled tibia homogenate, 4 ml of 100 000×g supernate of 10 day old embryo homogenate 0 25 M sucrose 0 05 M tris buff r pH 7 6 0 0[illegible] M KCl 1×10⁻³ M EDTA and 5×10 M FeSO in the final volume of 8 ml Sampl s were incubated at 37 for 1 h

system required the ferrous rather than ferric iron (Table 16) Adding cuprous ion or chelators for cuprous and cupric ions had no effect on hydroxylation

Requirement of other cations The addition of calcium magnesium cobaltous manganous or zinc ions did not increase the activity of hydroxylating system Requirement of potassium ion was demonstrated by Juva and Prockop (1966*b*) The effect of potassium ion was further tested and comparison was made to the effect of sodium ion

As shown in Table 17 addition of potassium chloride increased the hydroxylation Sodium ion also increased the activity but to a smaller degree At high concentrations both ions were inhibitory Comparison was made only between the amounts of potassium and sodium ions added to

Table 16 Effect of iron and copper chelators on the hydroxylation of solubili ed substrate

Experimental conditions	Total C dpm×10	Hypro C	
		dpm×10	% of total C
Expt 1			
Complete sy tem+FeSO	3 00	8 3	28
Complete system+FeSO +Tiron	3 00	[illegible]	2[illegible]
Complete system+FeCl+α α dipyridyl	3 2[illegible]	2 5	7 [illegible]
Complete system+o phenanthroline	3 56	2 6	7 3
Expt 2			
Complete system minus $FeSO_4$	4 90	3 6	7 5
Complete system minus $FeSO_4$+CuCl	5 30	3 9	7 4
Complete system+$FeSO_4$+Cupron	5 90	10 9	18
Complete system+FeSO +DDTC	5 40	10 0	19

The complete hydroxylating system was the same as described in Table 15 except that sucro e was omitted The concentrations of FeSO and FeCl were 5×10^{-4} M and of CuCl 1×10^{-4}M The concentrations of Tiron α α dipyridyl, o phenanthroline, Cupron DDTC (diethyldithiocarbamate) were 1 × 10 M

Table 17 Effect of potassium and sodium on the hydroxylation of olubili ed substrate

Concentration of ions added to hydroxylating system M				Hypro C % of total C
Expt 1	[K]	[Na]	[K + Na]	
	—	—	—	8 6
	0 02	—	0 0[illegible]	23
	0 2	—	0 [illegible]	22
Expt 2	—	—	—	13
	0 0[illegible]	—	0 02	21
	0	—	0 [illegible]	18
Expt 3	—	—	—	8 6
	0 0[illegible]	—	0 02	14
	—	0 0	0 0[illegible]	1[illegible]
	0 04	—	0 04	13
	—	0 04	0 04	10
	0 04	0 04	0 08	13
	0 1	—	0 1	13
	0 1	0 1	0 2	12
	0	—	0 2	12
	—	0 [illegible]	0 [illegible]	11
	0 6	—	0 6	5 9
	—	0 6	0 6	9 4

The hydroxylating sy tem was the ame as in Table 16 In expt 1 and 1 M KCl extract from a 100 000×g s liment of anaerobically labeled tibia homog nate was employed as sul trate *In expt 3 100 000×g supernate from the same tibia homogenate was employed*

the system The cation concentration of the embryonic supernate was not determined Definite conclusions about the specific requirement of potassium ion for hydroxylation cannot be drawn A more purified system is necessary to elucidate this problem

Some Characteristics of the Embryonic Supernate

As shown in Fig 8 the hydroxylation of solubilized substrate was completed in about 30 min at 37° The requirement of embryonic supernate is demonstrated in Fig 9 In this experiment when incubation time was 2 h 4 ml of embryonic supernate were required to obtain maximal hydroxylation Some variation was observed among different preparations of embryonic supernate The amount necessary for maximal hydroxylation varied between 3 and 6 ml of embryonic supernate It was further observed that supernate prepared from 10 day old embryos was more potent than those prepared from 7 8- or 9 day old embryos Supernates from older embryos tested up to the age of 13 days did not show any higher hydrox

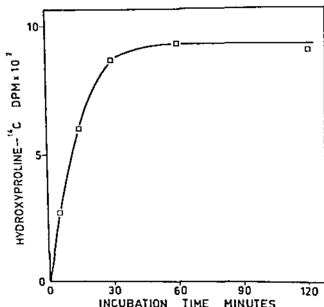


Fig 8 Rate of hydroxylation of solubilized substrate

Experimental conditions were the same as in Table 16 except that the concentration of F SO was 1×10 M and the samples were incubated for the periods indicated. The amount of hydroxyproline present in substrate preparation before hydroxylation was subtracted from the values obtained.

Table 16 Effect of iron and copper chelators on the hydroxylation of solubilized substrate

Experimental condition	Total ^{14}C dpm $\times 10^{-4}$	Hypro-^{14}C	
		dpm $\times 10^{-3}$	% of total ^{14}C
Expt 1			
Complete system + $FeSO_4$	3.00	8.3	2[illegible]
Complete system + $FeSO_4$ + Tiron	3.00	[illegible]	25
Complete system + $FeCl_3$ + α,α-dipyridyl	3.25	2.[illegible]	[illegible]
Complete system + o-phenanthroline	3.56	2.6	[illegible]3
Expt 2			
Complete system minus $FeSO_4$	4.80	3.6	[illegible]5
Complete system minus $FeSO_4$ + $CuCl_2$	5.3[illegible]	3.9	[illegible]4
Complete system + $FeSO_4$ + Cupron	[illegible].9[illegible]	10.9	18
Complete system + $FeSO_4$ + DDTC	5.40	10.0	19

The complete hydroxylating system was the same as described in Table 15 except that sucrose was omitted. The concentrations of $FeSO_4$ and $FeCl_3$ were 5×10^{-4} M, and of $CuCl_2$ 1×10^{-3}M. The concentrations of Tiron, α,α-dipyridyl, o-phenanthroline Cupron, DDTC (diethyldithiocarbamate) were 1×10^{-3}M.

Table 17 Effect of potassium and sodium on the hydroxylation of solubilized substrate

Concentration of ions added to hydroxylating system M				Hypro-^{14}C % of total ^{14}C
	[K]	[Na]	[K + Na]	
Expt 1	—	—	—	8.6
	0.02	—	0.02	23
	0.2	—	0.2	22
Expt 2	—	—	—	13
	0.02	—	0.02	21
	0.2	—	0.2	18
Expt 3	—	—	—	8.6
	0.02	—	0.02	14
	—	0.02	0.02	12
	0.04	—	0.04	13
	—	0.04	0.04	10
	0.04	0.04	0.0[illegible]	12
	0.1	—	0.1	13
	0.1	0.1	0.2	12
	0.[illegible]	—	0.2	12
	—	0.2	0.2	11
	0.6	—	0.6	5.9
	—	0.6	0.6	[illegible]4

The hydroxylating system was the same as in Table 16. In expts 1 and 2, 1 M KCl extract from a 10,000 [illegible] sediment of anaerobically labeled chick homogenate was employed as substrate. In expt 3 [illegible] 100,000 [illegible] supernate from the same chick homogenate was employed.

the system The cation concentration of the embryo ... determined Definite conclusions about the specific ... potassium ion for hydroxylation cannot be drawn A ... is necessary to elucidate this problem

Some Characteristics of the Embryonic Supernate

As shown in Fig 8, the hydroxylation of solubilized ... completed in about 30 min at 37° The requirement of embryonic ... is demonstrated in Fig 9 In this experiment when incub... 2 h, 4 ml of embryonic supernate were required to obtain max... ylation Some variation was observed among different ... embryonic supernate The amount necessary for maximal hydrox... varied between 3 and 6 ml of embryonic supernate It was furth... that supernate prepared from 10 day old embryos was more ... those prepared from 7, 8 or 9 day old embryos Supernates from ... embryos, tested up to the age of 13 days, did not show any h... ...

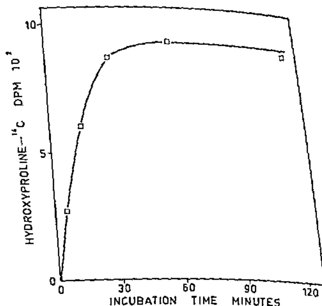


Fig 8 Rate of hydroxylation of solubilized substrate

Experimental conditions were the same as in Table 16 except that the concentration of $FeSO_4$ was 1×10^{-4}M and the samples were incubated for the periods indicated The amount of hydroxyproline present in substrate preparation before hydroxylation was substracted from the values obtained

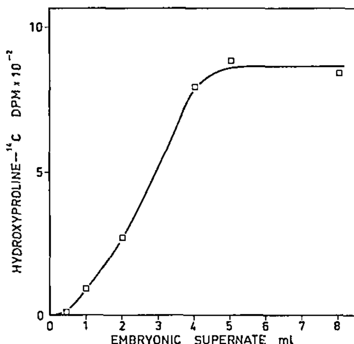


Fig 9 Requirement of embryonic supernate in hydroxylation

The experimental conditions were the same as in Fig 8 except that all samples were incubated for 2 h and the volume of embryonic supernate varied as indicated The amount of hydroxyproline present in substrate preparation before hydroxylation was subtracted from the values obtained

ylating activity During storage at —20° the hydroxylating activity of embryonic supernate decreased

The hydroxylating activity of embryonic supernate decreased rapidly during incubation at 37° (Fig 10) After varying incubation periods at 37°, substrate and ferrous iron were added and the incubation was continued for a period of 1 h The content of hydroxyproline ^{14}C observed in sample where the substrate and iron were added at zero time of preincubation was referred to as 100 The inactivation was most rapid in the beginning of the incubation After the preincubation period of 1 h embryonic supernate had lost most of its hydroxylating activity Considerable variation in the rate of inactivation was observed between different preparations

Hydroxylating capacity of embryonic supernate was tested by adding increasing amounts of substrate to the hydroxylating system as described in Table 16 Fig 11 shows that the synthesis of hydroxyproline ^{14}C was dependent on the amount of substrate added The correlation between the

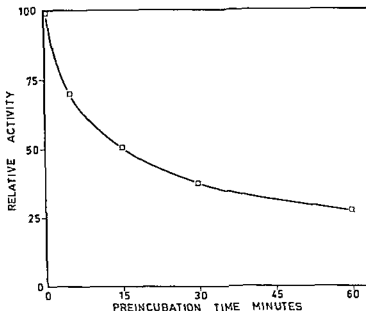


Fig 10 Inactivation of embryonic supernate

The experimental conditions were the same as in Table 16 except that labeled substrate and ferrous sulphate were added after the various preincubation periods indicated and the incubation was continued for 1 h The content of hydroxyproline in the sample in which the substrate was added at zero time of preincubation period is referred to as 100

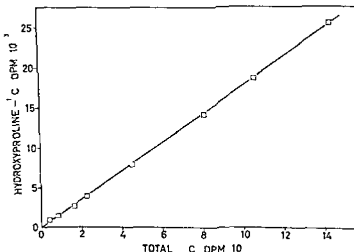


Fig 11 Effect of various amounts of solubilized substrate on the formation of hydroxyproline

Aliquots from a dialyzed 100 000 × g supernate of tibiae homogenate labeled with L proline C in the presence α α dipyridyl in aerobic conditions were added to the hydroxylating system described in Table 16

amount of substrate added and the synthesis of hydroxyproline was linear within the tested range To ensure the linear correlation between the amount of substrate added and the hydroxyproline formed the aliquots of substrate in the experiments were selected to contain 4000—40,000 dpm of peptide bound proline ^{14}C

Various Sources of Substrate for Hydroxylation

Preparations which served as substrates for hydroxylation could be obtained either by incubating collagen synthesizing tissues under nitrogen or in aerobic conditions by preventing the hydroxylation of proline with $\alpha\alpha'$ dipyridyl both *in vitro* and *in vivo* (Table 18) Preparation and

Table 18 Various sources of substrate for hydroxylation

Substrate preparation	Increase in Hypro ^{14}C
	% of total ^{14}C in sample
I Preparation labeled anaerobically *in vitro*	
Embryonic cartilage homogenate	
1200 × *g* pellet	2 6
1200—15 000 × *g* pellet	3 6
15 000—100 000 × *g* pellet	13
100 000 × *g* supernate	16
1 M NaCl extract of 1200—15 000 × *g* pellet	21
Hot water extract of 1200—15 000 × *g* pellet	19
1 M NaCl extract of 15 000—100 000 × *g* pellet	23
Embryonic skin homogenate	
1 M KCl extract	12
II Preparations labeled aerobically in the presence of $\alpha\alpha$ dipyridyl *in vitro*	
1 M KCl extract of cartilage homogenate	24
III Preparations labeled aerobically *in vivo*	
1 M KCl extract from 4-day embryo homogenate	12
1 M KCl extract from 10 day embryo labeled in the presence of α,α dipyridyl	33

Preparation of various substrates is described in the text The increase in Hypro C was calculated by subtracting the Hypro C content in the sample incubated at 4 from the Hypro C content in the sample incubated at 3

hydroxylation of substrates from embryonic cartilage are described earlier (pp 39, 42) The 1 M KCl extract of embryonic skin was prepared from the homogenate of skin slices of 10 day old embryos incubated under the same aerobic conditions as employed for the labeling of tibiae In aerobic conditions the substrate for hydroxylation was obtained from tibiae incubated as described in Table 6 The final concentration of α,α' dipyridyl was 1×10^{-3}M

A preparation labeled *in vivo* was made by placing 1 μCi of L proline ^{14}C in 0 5 ml of 0 15 M NaCl into the air sac of each of forty 4 day old chick embryos After incubation of 15 h the embryos were homogenized in 1 M KCl The homogenate was dialyzed and aliquots were added to the hydroxylating system Substrate for hydroxylation was prepared also by placing 1 56 mg α,α dipyridyl dissolved in 0 5 ml of 0 15 M NaCl into the air sac of 10 day old embryos After 1½ h 4 μCi L proline ^{14}C in 0 5 ml of 0 15 M NaCl was pipetted into the air sac and the incubation was continued for 2 h The embryos were homogenized in 1 M KCl and extracted by continuous shaking overnight at 4°, and thereafter centrifuged at 100,000$\times g$ at 4° for 1 h The supernate was dialyzed and aliquots were added to hydroxylating system

The values, quoted in Table 18 do not indicate the relative availability of collagen precursor on tissues, because the methods for preparing substrates varied Considerable variation was observed when particulate fractions of tibia homogenate were employed as substrate Two to fivefold increase in hydroxyproline ^{14}C was observed with most of the particulate preparations with some preparations no activity was observed especially after storage at —20° The 1 M KCl or 1 M NaCl extract from 15 000—100 000$\times g$ pellets of tibia homogenate gave the most constant results as judged by increase in hydroxyproline ^{14}C content It was found to be stable during storage at —20° for months

Hydroxylation of Solubilized Substrate in Partially Purified System

Partial purification of hydroxylating enzyme Homogenate from 10 day old chick embryos was centrifuged at either 15 000$\times g$ or 100 000$\times g$ at 4° for 1 h The supernate was adjusted to pH 5 0 with 0 5 M acetic acid and centrifuged at 100 000$\times g$ at 4° for 1 h The supernate from second centrifugation was re adjusted to pH 7 0 with 0 1 M potassium hydroxide The neutralized supernate had a hydroxylating activity comparable to that of the original embryonic supernate Solid ammonium

sulphate (ACS reagent Fisher Scientific Co) was slowly added to neutralized supernate with continuous stirring The final concentration was 25 % (w/v) The pH was checked continuously and adjusted back to pH 7 0 with 0 01 M potassium hydroxide when necessary The white precipitate was centrifuged at 15 000×g at 4° for 30 min The sediment was dissolved in 0 1 M potassium chloride solution the volume of which was one tenth

Table 19 Hydroxylation of solubilized substrate in partially purified system Effects of ethanol extract and ascorbate

Additions to system	Total C dpm × 10⁻⁴	Hypro C	
		dpm × 10⁻³	% of total C
Expt 1			
None	1 22	0 49	4 0
Enzyme	1 26	0 67	5 3
Enzyme + EE	1 14	1 05	9 2
Enzyme + EE + Ascorbate	1 1?	1 27	11
Expt 2			
None	1 24	0 31	2 5
Ascorbate	1 35	0 35	2 6
Enzyme + Ascorbate	1 21	0 91	7 5
Enzyme + Ascorbate + EE	1 31	1 85	14
Expt 3			
Ascorbate	1 01	0 6[illegible]	6 1
Enzyme + Ascorbate	1 09	1 23	11
Enzyme + EE + Ascorbate	1 18	1 60	14
Expt 4			
Enzyme	1 16	0 36	3 1
Enzyme + EE	1 11	0 [illegible]	[illegible] 0
Enzyme + 3 × EE	1 09	0 [illegible]6	7 0
Expt			
Embryonic supernate	1 05	2 56	24
Enzyme + EE	0 98	2 06	?1

EE = ethanol extract

Incubation mixture contained ... solubilized substrate from the same batch as indicated in Table 16 0 ? M KCl 0 05 M tris buffer pH 7 6 1 × 10 M EDTA, and 1 × 10 M FeSO in the final volume of 8 ml The enzyme was added in a volume of 0 5 ml The concentration of ascorbate was 1 × 10 M Ethanol extract (EE) was prepared by adding absolute ethanol into 5 ml of 15 000×g or 100 000×g supernate of embryo ... to the final concentration of 80 % (v/v) The suspension was centrifuged at 15 000 g at 4 for 20 min The supernate was evaporated to dryness in vacuo and the residue was dissolved in 4 0 ml of distilled water The samples were incubated ... f ... 1

of the original supernate All the operations were carried out in ice bath This preparation is hereafter referred to as (crude) enzyme The enzyme did not lose its activity at —20° during a storage period of 3 months

Hydroxylating activity of partially purified enzyme The enzyme alone had only little hydroxylating activity (Table 19) When ethanol extract from embryo homogenate was added to system further hydroxylation was observed Ascorbic acid potentiated the effect of the ethanol extract The activity of ethanol extracts varied considerably When the most active preparations were used an equal increase in hydroxyproline was observed as when whole embryonic supernate was employed About one third of ethanol extract preparations were inactive and ascorbate did not restore their activity A 10 min boiling did not inactivate the ethanol extract It could be adsorbed on charcoal and on Dowex 1 (chloride form) It was stable at pH 10 but not below pH 4 Reduced 2 amino-4 hydroxy 6 7 dimethylpteridine (Pohland *et al* 1951), lipoic acid glutathione NADH

*Table *0 Requirement of an additional cofactor*

Experimental conditions	Total ¹C dpm × 10⁻³	Hypro C dpm × 10 ³	Hypro C % of total C
Expt 1			
4 ml ES	8 60	1 93	2
1 ml ES	10 8	0 72	6 7
1 ml ES + ethanol extract	8 40	1 66	°0
1 ml ES + 5 ml boiled FS	10 4	1 03	9 9
Expt 2			
4 ml ES	8 00	1 S9	°4
1 ml ES	8 30	0 81	9 8
4 ml Dowex 1 treated ES	7 90	0 53	6 7
1 ml ES + 0 1 M KCl eluate from resin	9 10	1 13	1_
4 ml charcoal treated FS	9 40	0 69	7 3
1 ml FS + ethanol eluate from charcoal	10 0	1 29	13

ES is 100 000×*g* supernate from 10 day old embryo homogenate

The sul trate was from the same batch of preparation as indicated in Tabl 1(Ethanol extract was prepared as indicated in Table 19 Five ml of 100 000×*g* supernate of 10 day old embryo homogenate were passed through a Dowex 1 (chloride form) column (10 × 30 mm) and the column was eluted with 6 *ml of 0 1 M KCl* Five ml of the same embryonic supernate were shaken with 100 mg of charcoal for 10 min and centrifuged at 15 000×*g* for °0 min The charcoal was washed with 20 ml of absolute ethanol and the eluate was evaporated to dryness *in vacuo* The residue was dissolved in 4 ml of distilled water All operations were carried out at 4

Table 21 Effect of carbon monoxide on the hydroxylation

Gas mixture	Total C	Hypro C	
		dpm × 10	% of total C
Expt 1			
4 0 % O_2 + 96 % N_2	4 20	7 9	19
3 93 % O_2 + 46 9 % N_2 + 49 2 % CO	4 30	8 7	20
Expt 2			
In air	5 10	4 9	9 6
3 93 % O_2 + 46 9 % N_2 + 49 2 % CO	4 20	4 8	11

The hydroxylating system was the same as in Table 16 The experiment was performed in Warburg flasks The flasks were flushed with gas mixtures for 7 min and then incubated for 3 h at (See details in the text)

NADPH, or FAD, all of them in concentration of 1×10^{-4}M, neither replaced the ethanol extract nor improved the hydroxylating activity of partially purified system

Additional cofactor requirement Ethanol extract from embryo homogenate improved the hydroxylation when added to a system containing embryonic supernate in an insufficient volume to produce maximal hydroxylation (Table 20) Some increase in hydroxylation was observed when boiled embryonic supernate was added to a similar system Treatment with Dowex 1 (chloride form) inactivated the embryonic supernate and hydroxylation promoting factor was partly recovered *in 0 1 M potassium* chloride eluate from resin column Treatment with charcoal gave similar results The active component could be eluted from charcoal with ethanol diethyl ether or acetone Further purification and identification of the cofactors in ethanol extract were unsuccessful because the activity of the preparations varied in a marked degree and it was completely lost during further manipulations Also in this system attempts were made to replace the ethanol extract by reduced 2 amino 4 hydroxy 6 7 dimethylpteridine, NADPH NADH, FAD, glutathione, or lipoic acid, but none of these showed any effect

Various activators and inhibitors involving in the enzymatic reactions were also tested in the hydroxylating system described in Table 16 Glutathione mercaptoethanol p chloromercuribenzonate, iodoacetate, or fluoroacetate all of them in *concentration 1×10^{-3}M did not show any* effect on hydroxylation Potassium cyanide, in concentration 1×10^{-4}M, caused a slight inhibition probably due to the binding of ferrous iron

Table 22 Reactivation of embryonic supernate with ascorbic acid and α ketoglutarate

Preincubation min	Additions	Total C dpm × 10 ³	Hypro C dpm × 10 ³	Hypro C % of total ¹⁴C
Expt 1				
0	None	43 0	10 0	23
0	Ascorbate	49 2	10 2	21
30	None	41 0	4 10	10
30	Ascorbate	41 1	4 70	11
60	None	38 9	3 30	8 5
60	Ascorbate	41 1	3 60	8 8
Expt 2				
0	None	12 5	1 70	14
30	None	11 3	0 85	7 5
30	α KG*	10 0	1 64	16
Expt 3				
0	None	13 2	1 60	12
60	None	13 2	0 96	7 3
60	α KG	13 9	1 63	12
60	Ascorbate	13 4	0 90	6 7
60	α KG + Ascorbate	14 0	2 35	17

α KG = α ketoglutarate

The experimental conditions were the same as in figure 10 Concentration of ascorbate was 1×10⁻⁴ M and of α ketoglutarate 1×10 M The substrate for expt 1 was 1 M KCl extract from the 100 000×*g* sediment of tibia homogenate labeled with L proline C *in the presence of α α dipyridyl and for expt 2 and 3 dialyzed 100 000×g* supernate of the same homogenate

An attempt was made to determine whether the reaction involved the compound P-450 which has been implicated in several other hydroxylations (Cooper *et al* 1965) As shown in Table 21 the hydroxylation was not inhibited by carbon monoxide The 100 000×*g* supernate of embryo homogenate or partially purified enzyme had no spectral properties characteristic of P 450

During the course of this work evidence was presented that α ketoglutarate was required for the hydroxylation of peptide bound proline in a similar system (Hutton Tappel and Udenfriend 1966) This observation was confirmed in two different ways Attempts were made to reactivate the preincubated embryonic supernate (Fig 10) As shown in Table 22 ascorbate in the concentration of 1×10⁻⁴M did not restore the activity, but when α ketoglutarate was added to the final concentration of 1×10 ³M

Table 23 Effect of α ketoglutarate and ascorbate on the hydroxylating activity of embryonic supernate

Embryonic supernate ml	Additions	Total C dpm × 10⁻³	Hypro C	
			dpm × 10⁻²	% of total C
Expt 1				
4	None	11 3	12 0	11
1	None	10 5	2 1	2 0
1	α KG	9 10	7 7	8 5
1	α KG+ Ascorbate	14 5	15 8	11
Expt 2				
4	None	12 3	1[illegible] 0	14
1	None	13 7	[illegible] 8	4 2
1	α KG	12 9	[illegible] 0	5 4
1	α KG+Ascorbate	14 6	21 0	14

* α KG=α ketoglutarate

The experimental conditions were the same as in Table 16, except that the volume of embryonic supernate varied as indicated The concentration of α ketoglutarate was 1×10^{-3}M and of ascorbate 1×10^{-4}M The substrate was from the same batch of preparation employed in expt [illegible] and 3 in Table 22

hydroxylating system did show at least as high activity as the system where fresh embryonic supernate was employed Similar results were obtained when α ketoglutarate was added to the hydroxylating system which contained one fourth of the embryonic supernate used in control samples α ketoglutarate as ascorbate together could replace the most part of embryonic supernate (Table 23)

III THE SYNTHESIS OF COLLAGEN PRECURSOR AND THE HYDROXYLATING ACTIVITY IN GRANULATION TISSUE

Synthesis of Collagen Precursor

Granuloma slices were incubated as described on page 15 After the incubation the slices were homogenized into 1 M KCl solution which contained 0 01 M tris buffer pH 7 4 and kept overnight at 4° under continuous shaking The samples were centrifuged at 100 000×g at 4°

Table 24 Hydroxylation of the peptide precursor of collagen from granulomas of different ages

Age of granulomas days	Temperature C	Total C dpm × 10⁻⁴	Hypro C	
			dpm × 10⁻³	% of total C
Expt 1				
7	4	1 87	0 24	1 3
	37	1 66	2 30	14
12	4	1 56	0 28	1 8
	3	1 46	1 90	13
22	4	1 4[illegible]	0 85	5 9
	37	1 37	1 45	11
29	4	1 60	0 [illegible]5	4 7
	37	1 64	2 30	14
Expt 2				
7	4	2 23	0 20	0 9
	37	2 12	2 15	10
13	4	2 66	0 45	1 7
	37	2 38	[illegible] 65	11
28	4	1 58	0 25	1 6
	37	1 43	1 45	10

Samples contained 5 ml of 1 M KCl extract from granuloma slices labeled *in vitro* with L-proline C in the presence of α α dipyridyl 5 ml of 100 000×*g* supernate from 10 day-old chick embryo in a final volume of 15 ml. Otherwise the incubation mixture was the same as in Table 16 The samples were incubated for 1 h

for 1 h and the supernate was dialyzed against running tap water over night with 100 mg of L proline Aliquots from retentate were employed as a substrate

When the hydroxylation was inhibited with α α dipyridyl during the labeling of slices a precursor of collagen was synthesized (Table 24) The hydroxyproline content in the final trichloroacetic acid extract was the same as in the 1 M KCl extract from granuloma slices which were incubated without α α dipyridyl and processed similarly (Table 25) No attempts were made for quantitative assay of nonhydroxylated material When 1 M KCl extract from granuloma slices incubated without α α dipyridyl was used as a substrate for hydroxylation an increase in hydroxyproline was observed (Table 25) The observed increase small but constant suggested that in granulation tissue there is a pool of peptide precursor of collagen

Table 25 Hydroxylation of 1 M KCl extract from normal granulomas labeled with L proline C in vitro

Age of granulomas days	Temperature C	Total C dpm × 10⁻⁴	Hypro C	
			dpm × 10⁻³	% of total C
Expt 1				
7	4	1 43	1 71	12
	37	1 26	1 76	14
12	4	1 51	1 88	12
	37	1 32	2 01	15
22	4	1 14	1 31	11
	3	1 23	1 49	12
29	4	1 27	1 43	11
	37	1 13	1 52	13
Expt 2				
7	4	2 42	3 30	14
	37	2 06	3 50	17
13	4	3 62	6 20	17
	37	2 94	6 25	21
28	4	2 22	2 54	11
	37	1 83	2 58	14

The samples contained 1 M KCl extract from granuloma slices labeled with L proline C *in vitro* Other conditions were the same as in Table 24

The Hydroxylating Activity in Granulation Tissue of Different Age

Twenty four granulomas of the same age were pooled and homogenized in a Sorvall Omni Mixer for 1 min The homogenate was centrifuged at 45 000×g at 4° for 1 h and the supernate was used in hydroxylating system (Fig 12)

The hydroxylating activity of granuloma supernate was dependent on the age of granulation tissue As shown in Fig 12 the maximum activity was observed in about 20 day old granulomas Younger and older granulomas showed lower activity Compared to the rate of collagen synthesis in similar granulomas (Lampiaho and Kulonen 1967) a parallelism of these two functions is apparent It can be concluded that hydroxylating activity in granulation tissue reflects the activity of collagen synthesis

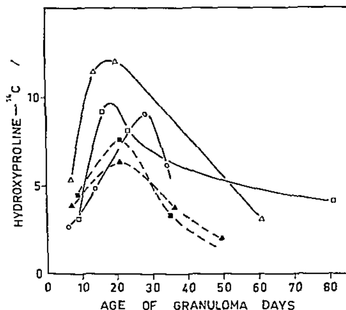


Fig 1 Hydroxylating activity in granuloma supernates of different age*

The hydroxylating mixtures contained as a substrate aliquots from a 100 000×g supernate from chick embryo tibia homogenate labeled with L-proline C in the presence of $\alpha\alpha$ dipyridyl, 0 05 M tris buffer pH 7 6 0 02 M KCl 1×10^{-3}M EDTA, 5×10^{-4}M FeSO and 10 ml of granuloma supernate in a final volume of 15 ml The samples were incubated at 37 for 1 h

DISCUSSION

Characteristics of chick embryo tibiae

Chick embryo tibiae were found to be very suitable for the studies on collagen biosynthesis. Dissection of tibiae in large quantities caused no special difficulties. When L-proline-^{14}C was employed as a labeled precursor, 21—25 % of the incorporated total ^{14}C was consistently recovered as hydroxyproline-^{14}C. Assuming that an equal amount of ^{14}C was incorporated into the hydroxyproline-^{14}C and proline-^{14}C in collagen, the result suggests that about one half of the proline-^{14}C which incorporated into nondialyzable forms was in collagen.

An increase in the rates of proline incorporation and hydroxyproline synthesis was observed with a concomitant decrease in sulphate and uridine incorporation. Similar increase in collagen synthesis has been observed in sponge biopsy connective tissue by Kao *et al.* (1963). An indisputable explanation for the observed transient increase in the rate of collagen synthesis cannot be given.

The subsequent decrease, especially in hydroxyproline synthesis, could be accounted for by the lack of nutritients in the medium. The importance of ascorbic acid for the synthesis of collagen in tibiae in tissue culture conditions has been demonstrated by Jeffrey and Martin (1966*a*, *b*).

The rate of incorporation of uridine-2-^{14}C into nondialyzable form was at its highest in freshly isolated tibiae. The result indicated that the rate of RNA synthesis decreased rapidly at the time when the rate of protein synthesis was increasing. The reason for this difference is not readily apparent.

The observation that sulphate incorporation was decreasing during the period when the labeling of proteins was increasing suggested the independence of the synthesis of sulphate acid mucopolysaccharides and collagen. Further support favoring this concept has recently been presented by Bhatnagar and Prockop (1966).

The absolute dependence of the collagen synthesis on oxygen has been

reported among others by Kao et al (1963) They demonstrated that nitrogen atmosphere in sponge biopsy connective tissue abolished both the incorporation of proline and the synthesis of hydroxyproline In chick embryo tibiae anaerobic conditions strongly inhibited the synthesis of hydroxyproline while the incorporation of proline continued The results suggested that in the biosynthesis of collagen peptide synthesis and hydroxylation of proline are separate functions This was further supported by the effect of ferrous iron chelator, α α′ dipyridyl, on the incorporation of proline and the synthesis of hydroxyproline in tibiae

The effect of different homogenizing techniques on the distribution of label among subcellular fractions emphasizes the precautions which must be taken into account in applying simple fractionation procedures to collagen synthesizing tissues Collagen forms characteristically large aggregates, and newly synthesized collagen is rapidly converted into forms which are progressively more difficult to disaggregate and to extract as soluble collagen (Jackson and Bentley 1960) The protein bound hydroxyproline in the 100 000×g sediment has been shown to have a higher specific activity than other fractions of tissue homogenates which indicates that this fraction represents a precursor of more rapidly sedimenting collagen (Eastoe 1961 Green and Lowther 1959 Lowther Green and Chapman 1961 Prockop Peterkofsky and Udenfriend 1962) The progressive accumulation of radioactive hydroxyproline suggested that the 100 000×g sediment was contaminated with more mature collagen Similar accumulation of proline into rabbit embryo skin microsomes has been reported by Bekhor and Bavetta (1965)

Effects of puromycin and chloramphenicol

The disproportionate inhibition of the synthesis of protein bound hydroxyproline by puromycin cannot be reconciled with the hypothesis that hydroxylation of proline precedes its incorporation into peptide unless it is assumed that puromycin produces some special effects in cartilage that have not been encountered in other systems However the effect of puromycin on the distribution of nondialyzable label among homogenate fractions and a comparison with the effects of chloramphenicol suggest that the mechanism by which protein synthesis was inhibited was similar to the specific mechanism demonstrated in bacteria It was reported by Prockop and Juva (1965b) that puromycin in concentration of up to 100 μg/ml did not inhibit the hydroxylating reaction when a proline rich polypeptide precursor of collagen was employed as a substrate for hydrox

ylation Also labeled polypeptides which continued to be synthesized in the presence of puromycin were considerably smaller than the polypeptides recovered from control samples (Juva and Prockop 1966c) The experiments with tritiated proline and ^{14}C labeled amino acid mixture also suggested that the polypeptides synthesized in the presence of puromycin had an amino acid composition similar to those synthesized in control samples The observations support the conclusion that puromycin prematurely terminated the synthesis of polypeptide precursor of collagen, and that when large concentrations of puromycin were present, the polypeptides which continued to be synthesized were of insufficient size to serve as substrates for the hydroxylating reaction

Recently Manning and Meister (1966) reported that when the incorporation of proline ^{14}C into collagen in carrageenin granuloma slices was inhibited by about 90 % with puromycin the formation of tritiated water, used as an index for hydroxylation, was inhibited by only about 50 % This suggests that hydroxylation of proline continues even when the synthesis of collagen is markedly decreased They also showed that puromycin inhibited the incorporation of proline into collagen proline and collagen hydroxyproline to about the same extent The activity of microsomally bound hydroxyproline insoluble in cold trichloroacetic acid was markedly reduced in the presence of puromycin, in contrast to the activity of soluble peptide bound hydroxyproline which was disproportionally higher

Manner and Gould (1963) have shown that the formation of free hydoxyproline was essentially normal in chick embryos whose collagen synthesizing ability had been almost completely suppressed by puromycin During the procedure employed in the present work free hydroxyproline and small hydroxyproline containing peptides were lost during the dialysis of the subcellular fractions of embryonic tibiae

The biological significance of free hydroxyproline and hydroxyproline containing small peptides is not thoroughly known It is generally accepted that free hydroxyproline cannot be utilized for the synthesis of collagen The strongest evidence supports the concept that free hydroxyproline and small hydroxyproline containing peptides represent degradation products of newly formed collagen (*e g* Hurych and Chvapil 1965*b*) On the other hand the specific activity of free hydroxyproline excreted in the medium where costal cartilage of rat were incubated with proline ^{14}C, was 20 times higher than that of hydroxyproline in soluble collagen (Daughaday and Mariz 1962*a*) Possibly free hydroxyproline and small hydroxyproline containing peptides do not reflect only the degradation of collagen, but also

the synthesis and degradation of hydroxyprolyl sRNA and of peptides which are hydroxylated prematurely i e at the stage where the peptide chains are still of insufficient size for the formation of collagen molecule

Hydroxylation of peptide bound proline

The substrate for the hydroxylation cannot be free proline since the substrate was initially isolated in a particulate form the hydroxylating system did not require ATP, and the conversion of labeled proline in the substrate to hydroxyproline ^{14}C occurred at over a thousand times the rate of conversion of free proline-^{14}C to hydroxyproline ^{14}C in the same system The substrate is unlikely to consist of prolyl sRNA since it was stable to alkali and ribonuclease, and when solubilized by extraction with 1 M sodium chloride it separated completely from nucleic acid by density gradient centrifugation The relative sensitivity of the substrate to chymotrypsin trypsin and collagenase was similar to that of collagen in which the secondary structure has been bestroyed (Harrington and Hippel 1961) The recovery of fragments after treatment with trypsin and chymotrypsin which were nondialyzable but which no longer served as substrates also suggested that the substrate for hydroxylation is relatively large polypeptide

The anaerobically labeled particulate fractions contained both the substrate and the enzyme The heat stable factor contributed by the embryonic supernate was lost after dialysis Addition of ferrous iron increased the activity of the system in a marked degree which has further confirmed when solubilized substrate was used as a precursor for hydroxyproline

Similar results were recently reported by Hutton Tappel and Udenfriend (1967) Involvement of cuprous ion in hydroxylation has been suggested by Chvapil *et al* (1966) but in the system employed, this could not be demonstrated Various other metal ions have been tested but none of these could replace iron (Prockop and Juva 1965*b* Hutton Tappel and Udenfriend 1967) The fact that ferrous iron is involved in the hydroxylation of proline has made it possible to prepare peptide precursor of collagen in aerobic conditions (Juva and Prockop 1966*b*, Hutton and Udenfriend 1966 Lukens 1966)

To obtain maximal hydroxylation of solubilized substrate relatively large amounts of embryonic supernate were required Apparently the amount of enzyme was not a limiting factor in the system because major part of embryonic supernate could be replaced by ascorbate and α ketoglutarate The rapid inactivation of embryonic supernate was apparently

dependent on the disappearance of cofactors and not on the denaturation of the hydroxylating enzyme, because by adding α ketoglutarate and ascorbate initial activity was obtained

The enzyme required for hydroxylation was partly purified and by itself showed only minimal hydroxylating activity By adding ethanol extract from embryonic supernate hydroxylation was observed which was potentiated by ascorbate A 200 fold purification of the enzyme has recently been reported by Kivirikko and Prockop (1967c) The active component of ethanol extract is most likely to be α ketoglutarate (Hutton, Tappel and Udenfriend 1966, 1967 Kivirikko and Prockop 1967b)

The requirements for ferrous ion and ascorbate are consistent with the participation of a transition metal ion and a reducing compound in several other mono oxygenase reactions (Hayaishi 1964 Friedman and Kaufman 1966) The stimulatory effect of α ketoglutarate in hydroxylation is of great interest being a key intermediate in tricarboxylic acid cycle Another intermediate of this cycle fumarate is involved in the β hydroxylation of dopamine (Levin Levenberg and Kaufman 1960) Hutton Tappel and Udenfriend (1967) reported that no correlation was observed between the amount of α ketoglutarate which disappeared and the amount of substrate hydroxylated

Hydroxylating activity in granulation tissue of different age

Sponge granuloma tissue offers an attractive model system for studies on the regulation of collagen biosynthesis In the life cycle of granulation tissue there are three distinct phases (1) proliferation of the fibroblasts during the phase synthesis of collagen is low (2) an active phase of collagen synthesis and (3) involution (Kulonen 1965) The hydroxylating activity was on its highest in about 20 days old granulomas It is of great interest that the rate of collagen synthesis in similar granulomas was shown to have its maximum at about the same age (Lampiaho and Kulonen 1967) Stoichiometric comparation between the rate of peptide synthesis and the hydroxylating activity was not possible because neither characteristics of substrate for hydroxylation nor the conditions for the assay of hydroxylating activity are known in details well enough the parallelism is, however, obvious

The peptide precursor of collagen synthesized in chick embryo tibiae could be hydroxylated with the system which contained rat granuloma supernate and *vice versa* Hydroxylating activity in soluble preparations

of fetal rat skin, adult rat liver and guinea pig granulation tissue has been reported by Hutton and Udenfriend (1966), and in several tissues of the adult rat (Juva 1967). This suggests that the mechanism for collagen synthesis in different animals is actually similar. A slight but constant increase in hydroxyproline-^{14}C was observed when the 1 M KCl extractable material from granulomas labeled with L-proline-^{14}C *in vitro* was employed as a substrate. The results, not conclusive, however suggested that in normal tissues there is a pool of peptide precursor of collagen, which is not hydroxylated.

The factors regulating collagen biosynthesis are poorly known, which is a consequence of the lack of knowledge about the synthesis of collagen molecule. It is obvious that in the biosynthesis of collagen bound hydroxyproline and hydroxylysine two processes are involved: (1) synthesis of polypeptide and (2) hydroxylation of peptide bound proline and lysine. The first process is most likely to be similar to that in other proteins. The hydroxylation reaction might prove to be a site of regulation in the biosynthesis of collagen.

The substrate, a proline and lysine rich polypeptides for which a name protocollagen has been suggested (Juva and Prockop 1965), must apparently be of considerable molecular size (Prockop and Juva 1965*b*, Lukens 1966, Kivirikko and Prockop 1967*a*). When the growing polypeptide reaches a definite size and contains appropriate amino acid sequence which can be recognized by hydroxylating enzyme, proline and lysine are hydroxylated. Atmospheric oxygen is required for hydroxylation. When the supply of oxygen is limited the hydroxylation cannot occur and protocollagen accumulates inside the cells and no collagen is synthesized. Similar accumulation was also observed when hydroxylation was prevented by binding ferrous iron with α α'-dipyridyl (Juva *et al.* 1966). Ascorbate might be a limiting factor too. In scorbutic tissue hydroxyproline synthesis was inhibited disproportionally more than the total proline incorporation (Stone and Meister 1962*a*). One cannot exclude the possibility that protocollagen accumulates in scurvy and hydroxylation is a limiting factor for collagen synthesis because of the ascorbate deficiency. Hutton, Tappel and Udenfriend (1967) have suggested that α-ketoglutarate might function as an allosteric activator of hydroxylating enzyme and its concentration in the cell could regulate the activity of this enzyme and thus the synthesis of collagen.

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ERRATA

Page 16 line 14 *reads* Reichardt
should be Reichard

Page 26 line 22 *reads* Zilversmuth
should be Zilversmit

Page 41 line 4 *reads* like
should be than

Pages 48 and 49, Table II and IV *reads* magnesium
should be natrium

Page 64, line 28 *reads* Pierce
should be Pearce

Page 68, line 13 *reads* Zilligen
should be Zilliken

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UNIVERSITY OF TURKU TURKU FINLAND

THE MUCOPOLYSACCHARIDES IN AGEING EXPERIMENTAL GRANULATION TISSUE

BY

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PREFACE

The present study was carried out at the Department of Medical Chemistry, University of Turku.

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Turku, January 1968

Aapo Lehtonen

CONTENTS

INTRODUCTION

The carbohydrate components of connective tissue comprise acid mucopolysaccharides (AMPS) and glycoproteins which are components of the amorphous ground substance which is interspersed between the fibres and the cells Sylven (1941) observed metachromasia in regenerating tissue before any fibres could be discerned The concentration of sulphated mucopolysaccharides (MPS) was highest during the period of maximal growth of the tissue and Sylven suggested that sulphated MPS are present already when new tissue is being formed Metachromasia and hexosamine content determinations (Dunphy and Udupa 1955) and increased synthesis of MPS (Layton 1950) have later shown that the content of MPS is high in the amorphous matrix in early stages of tissue repair

There are many investigations that show that the composition of AMPS changes in ageing cartilage (Kaplan and Meyer 1959), intervertebral discs (Hall *et al* 1957) aorta (Bertelsen and Jensen 1960) and skin (Loewi and Meyer 1958) Because AMPS of connective tissue are involved in a number of physiological processes including regulation of water content and electrolytes in extracellular fluids calcification and lubrication (Dorfman 1958) and because macromolecular aggregates of MPS linked to fibrillar collagen impart to the tissues their characteristic toughness and flexibility (Mathews and Lozaityte 1958) the changes in the MPS are of considerable interest Houck and Jacob (1958) suggested that the rate of formation of the MPS may be a critical feature in the ageing process and Sobel and Marmorston (1956) suggested that the ratio of hexosamine to collagen might be taken as a measure of biochemical age'

The purpose of this study was to determine the changes in the MPS in granulation tissue produced experimentally by the sponge implantation method A gas chromatographic method developed previously was used for the qualitative and quantitative analysis of the monosaccharide components of the MPS Because most methods of MPS fractionation usually require a large amount of MPS a microfractionation method involving electrophoresis on cellulose acetate was extensively employed

REVIEW OF THE LITERATURE

OCCURRENCE OF MUCOPOLYSACCHARIDES (MPS) AND THEIR VARIATION WITH AGE

The mucopolysaccharides of granulation tissue

Mucopolysaccharides in tissue repair One of the features of connective tissue repair in wounds is the appearance of amorphous material before any fibres can be discerned This material stains metachromatically with methylene blue and toluidine blue and has been thought to consist of MPS Sylvén (1941) observed metachromasia in healing wounds and thought sulphated MPS to be present in the process of healing The metachromasia rises to a peak on the fifth and sixth day and then declines (Dunphy and Udupa 1955 Kodicek and Loewi 1955 Penney and Balfour 1949) The increase in MPS is associated with intense proliferation of fibroblasts After the appearance of the collagen network the content of MPS decreases (Vinogradov 1966) An accelerated synthesis of MPS in regenerating tissue is revealed by the content of absorbed inorganic sulphate (Layton 1950 Layton *et al* 1958 Campani *et al* 1960 Kodicek and Loewi 1955) The high content of hexosamine has also been considered supporting evidence for the presence of MPS in wound healing (Dunphy and Udupa 1955 Ahmad 1961) Meyer (1947) proposed that young fibroblasts secrete hyaluronic acid chondroitin sulphate and a precursor of collagen into the surrounding tissue space Jackson *et al* (1960) found that most of the hexosamine in connective tissues of granulating wounds is associated with serum mucoproteins rather than with MPS in the wound exudate White *et al* (1961) suggested that both glycoproteins and MPS are essential components of the intercellular ground substance

The mucopolysaccharide content rapidly increases in the callus before a rise occurs in the collagen content (Kasavina and Zenkevich 1961, Udupa and Prasad 1963) The glucosamine content is twice as high as that of galactosamine in normal bone while approximately equal amounts of both amino sugars are found in fractures (Zenkevich and Kasavina 1962) Hyaluronic acid and chondroitin sulphates A and C have been found in fracture callus (Antonopoulos *et al* 1965 Solheim 1965 and 1966), and chondroitin sulphates were found in the greatest amounts together with hyaluronic acid in the relatively less mineralized parts of the callus (Solheim 1966)

Experimental granulomas The sponge implantation method which was first employed by Boucek and Noble (1955) is a valuable technique for reproducible sampling of connective tissue at any desired stage of development The variations with time under given experimental conditions can be closely followed, and adequate samples can be obtained for several biochemical determinations Boucek and Noble (1955) and Hruza and Hlavackova (1963) used nonresorbable polyvinyl sponge implants and Viljanto and Kulonen (1962) used viscose cellulose sponge implant Viscose sponge proved to be a good implantation material and Viljanto (1964) used it in his study of

the basis of wound healing Granulomas produced by introducing various irritants such as carrageenin (Robertson and Hinds 1956 Slack 1958, Chvapil and Cmuchalova 1961) oil of turpentine (Trnavsky et al 1961, Berenson and Dalferes 1960) alginic acid (Kimoto et al 1960) and silica powder (Collet and Daniel Moussard 1958 Daniel Moussard and Quesson 1961) have also been studied White et al (1961) used stainless steel mesh cylinders as implants

Boucek and Noble (1955) found that the chemical composition of tissue changes with age An early rapid synthesis of polysaccharides has been found to occur (Slack 1957 and 1958, Chvapil and Cmuchalova 1961 Trnavsky et al 1961, Collet and Daniel Moussard 1958 Daniel Moussard and Quesson 1961), while collagen fibres are formed later (Woessner and Boucek 1961 Kao et al 1957) The ratio of hexosamine to hydroxyproline diminishes with age in granulomas (Hruza and Hlavackova 1963 Kimoto et al 1960) In the early stage of tissue formation the hexosamine level parallels that of desoxyribonucleic acid but later the content of desoxyribonucleic acid decreases more rapidly than the content of MPS (Chvapil and Cmuchalova 1961) Robertson and Hinds (1956) Noble and Boucek (1958) and Daniel Moussard and Quesson (1961) found that the saline soluble hexosamine content first increases and then decreases in growing granulomas whereas the saline insoluble hexosamine content increases later Because the hexosamine fraction, especially the readily soluble fraction consisted mostly of glucosamine hyaluronic acid was thought to be the predominating MPS in young granuloma (Noble and Boucek 1958 Kimoto et al 1960 White et al 1961) Noble and Boucek (1958) noted that galactosamine appeared first in the saline insoluble fraction and then in the saline-soluble fraction Bollet et al (1958) analysed the MPS components in polyvinyl sponge granulomas and found evidence for the presence of three types of components They concluded that the content of chondroitin sulphate B increases in older granulomas Berenson and Dalferes (1960) identified four AMPS namely hyaluronic acid heparitin sulphate and chondroitin sulphates A and B in granulation tissue and found that the chondroitin sulphate content increases more rapidly than the hyaluronic acid content The amount of acid mucopolysaccharides increases more slowly than the amount of glycoproteins in granulomas (Delaunay and Bazin 1964) Jackson et al (1960) found that most of the bound hexosamine appeared to be associated with extracted proteins

The Changes in Mucopolysaccharides in Ageing Connective Tissue

In the course of its foetal and postnatal development the intercellular substance of connective tissue undergoes certain changes which are called maturation In recent years it has frequently been suggested that also the MPS of tissues change with age The polysaccharide patterns of cartilage aorta and skin at various ages have frequently been investigated

Cartilage

Loewi (1953) found that the content of chondroitin sulphate in tissues and its degree of polymerization are inversely proportional to age A decrease in the content of chondroitin sulphate in ageing cartilage was observed also by Shetlar and Masters

(1955) while the data of Miles and Eichelberger (1964) demonstrate that only minimal changes occur in the MPS composition of cartilage with age Kaplan and Meyer (1959) isolated the MPS of cartilage and found that the level of keratosulphate increases and that of chondroitin sulphate decreases in ageing cartilage Chondroitin sulphate A was replaced by chondroitin sulphate C Similar results were obtained by Lash and Whitehouse (1960) and Mathews and Glagov (1966) Seno *et al* (1965) isolated from old human rib cartilage an oversulphated keratosulphate fraction which was closely associated with chondroitin sulphate C

Changes in acid mucopolysaccharides similar to those occurring in hyaline cartilage occur in human intervertebral discs with age The discs consist of fibrocartilage (annulus fibrosus) surrounding a gel (nucleus pulposus) rich in AMPS Hall *et al* (1957) identified hyaluronic acid keratosulphate and chondroitin sulphate in the nucleus pulposus and concluded that the level of each undergoes a gradual change when the nucleus pulposus undergoes drastic transformation from a gelatinous fluid at birth to a desiccated mass in old age The keratosulphate content of the intervertebral discs rises with age (Hallen 1962 Buddecke and Sziegoleit 1964), whereas the ratio of chondroitin sulphates A and C decreases (Buddecke and Sziegoleit 1964) The total content of AMPS and the total content of chondroitin sulphates in the intervertebral discs decrease with age (Antonopoulos 1965 Solheim 1966 Davidson and Small 1963) Stockwell (1965) suggested that the territorial increase of keratosulphate in the deepest parts of cartilage may be related to the efficiency of nutrition in these areas as much as to their age Davidson *et al* (1961) studied the metabolism of MPS and concluded that the formation of chondroitin sulphate diminishes considerably in ageing cartilage

Aortic tissue

The age related changes and disorders of the arterial system have engendered much interest The relative amount of AMPS in dry arterial tissue decreases with age but the absolute amount appears to increase or remain constant (Bertelsen and Jensen 1960 Bertelsen 1962) The acid mucopolysaccharide content has been stated to increase more than the glycoprotein content (Bertelsen 1960) Clausen (1962) reported that the hexosamine content decreases with age as does also the ratio of hexosamine to hydroxyproline Hyaluronic acid and chondroitin sulphate decrease with age while chondroitin sulphate B and heparitin sulphate increase (Kaplan and Meyer 1960) The decrease of hyaluronic acid and the increase of chondroitin sulphate B have been confirmed by many investigators (Bertelsen and Jensen 1960 Zugibe 1962 Clausen 1963, Manley 1965) The decrease of hyaluronic acid and the increase of chondroitin sulphate in heart valves with age parallel their variation in the aorta (Moretti and Whitehouse 1963) The matrix properties of ageing arterial wall have been extensively reviewed by Milch (1965)

Skin

It was found by Loewi and Meyer (1958) that embryonic skin contains only small amounts of chondroitin sulphate B but relatively large amounts of hyaluronic acid and chondroitin sulphate C while adult skin contains more chondroitin sulphate B than hyaluronic acid The amount of heparin is larger in young than in old animals (Schiller and Dorfman 1960) Houck *et al* (1961) found neutral salt insoluble hexosamine in

skins of rats weighing more than 206 g and Houck and Jacob (1958) determined the hexosamine and collagen contents of rat skin and concluded that the decreasing ratio of hexosamine to hydroxyproline indicates that the formation of MPS is a critical feature of the ageing process

Other tissues

The highest specific activities of sulphate S^{35} after intraperitoneal dosage have been observed in MPS isolated from tissues of very young rats (Dziewiatkowski 1954) It was suggested by Gersh and Catchpole (1949) that the degree of polymerization in the ground substance varies with age Smits (1957) determined the hexosamine and hydroxyproline contents of bovine cornea, sclera tendon and skin As a general rule, the hexosamine content decreased in the course of development while the hydroxyproline content increased The ratio of hexosamine to collagen in femurs of rats decreases with age (Sobel *et al* 1954) The content of MPS in human spleens decreases with age but the MPS consistently contain about 25 % heparitin sulphate (Bitter and Muir 1966) Gessner and Bostrom (1965) identified chondroitin sulphate and heparitin sulphate in the cardiac jelly and suggested that MPS may be important in the critical stage of cardiogenesis

Mucopolysaccharides and collagen fibres

The nature of the interaction of MPS and collagen in connective tissue and its physiological significance have been subjects of major interest Gross *et al* (1952) found that hyaluronic acid chondroitin sulphate and heparin produce fibrous precipitates directly when they are added to collagen solutions Chondroitin sulphate plays an essential part in the development of the primary fibril aggregates and the depolymerization of chondroitin sulphate can be followed by studying its ability to produce fibrils in collagen solutions (Németh Csoka 1960 and 1961, Keech 1961) Dissolved collagen is precipitated by chondroitin sulphate in vitro (Gross 1958) and the precipitate obtained on adding MPS in acetate buffer to a collagen solution shows the typical collagen structure (Clerici *et al* 1962) Wood (1960) stated that chondroitin sulphates A and C accelerate fibril formation markedly whereas chondroitin sulphate B and hyaluronic acid have no effect Precipitation of collagen by MPS does not however constitute evidence for their participation in fibrinogenesis in vivo Gross (1956) found that typical collagen fibrils may be precipitated in the absence of uronic acid and a host of seemingly unrelated compounds also precipitate collagen fibrils

Jackson (1953 and 1954) found that treatment with hyaluronidase decreases the stability of tendon and suggested that MPS are important in the stabilization of collagenous fibres but this was denied by Embinder and Schubert (1951) and Partington and Wood (1963) The effect of AMPS on collagen is ascribed to electrostatic factor (Elden 1964 Woodin and Boruchoff 1955) Banga and Balo (1960) Balo *et al* (1960) and Kuhn (196) suggested that a neutral polysaccharide is one of the factors stabilizing collagen fibres Mathews and Lozaityte (1958) and Mathews (1965) found that the stability of the complexes formed by a MPS with collagen increases with increasing chain length of the mucopolysaccharide and presented a schematic model for the interaction of collagen and chondroitin sulphate protein macro

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molecules Loewi and Meyer (1958) suggested that the appearance of mature coarse collagen fibres is connected with the presence of chondroitin sulphate B and that chondroitin sulphate C is associated with young fine collagen fibres Zugibe (1962) found that the increase in the number of coarse collagen fibres paralleled an increase in the ratio of chondroitin sulphate B to chondroitin sulphates C and A

MPS may provide the necessary viscous medium required to keep the precipitated fibrils localized in specific areas and arranged in a particular pattern, the fibrils might otherwise precipitate in a random tangled mass around the cells (Gross 1956) The hypothesis that polar polysaccharide-protein polymers might be involved in the oriented formation of collagen fibres has been suggested by several authors (Partridge 1948, Jackson 1953 Kodicek and Loewi 1955)

That MPS and collagen have a different metabolism was shown by Bhatnagar and Prockop (1966) who were able to inhibit the synthesis of MPS without affecting the extrusion of collagen into the extracellular matrix They suggested that the formation of a complex between collagen and MPS is not a necessary step for the incorporation of collagen into the extracellular matrix

ANALYSIS OF MUCOPOLYSACCHARIDES

Extraction of mucopolysaccharides

The principal problem encountered in the isolation of pure MPS concerns the removal of bound protein under conditions that do not significantly degrade the polysaccharides The employed methods have been reviewed by Scott (1960) The isolation methods may be divided into methods in which strong extracting solvents and methods in which weak solvents are used

Water and salt solutions

Hyaluronic acid is more readily extracted than chondroitin sulphate from most tissues and is generally believed to be present only in the ground substance and not attached to the fibres (Meyer 1954) Some investigators have attempted to distinguish between the carbohydrates which are present mainly as constituents of the interfibrillary matter of tissues and those which are in some way combined with the fibres When Houck and Jacob (1960) extracted rat skin with sodium chloride solution about one third of the hexosamine remained insoluble and the authors suggested that this fraction was intimately associated with the tissue residue Using the same extraction method, Houck *et al* (1961) found that there was an abrupt appearance of insoluble hexosamine in the skins of rats with body weights exceeding 200 g and the insoluble hexosamine content continued to increase with age Mathieson and Pearce (1963) isolated hyaluronic acid from a saline extract of fat free rat skin in better than 80 % yield the product was uncontaminated by chondroitin sulphate B They also found that about two thirds of the galactosamine was insoluble and more hexosamine was removed from wet macerated hide than from acetone dehydrated hide Sweeny *et al* (1963) found that the fraction extracted by 0 15 N sodium chloride from rat skin consisted primarily of proteins but contained also glycoproteins and MPS

Certain MPS can be extracted from tissues with water, but they may be considered exceptional Malawista and Schubert (1958) described a method by which the chondro mucoprotein of nasal cartilage can be extracted with water in a yield that accounts for 80 per cent of the total hexosamine in the cartilage Shatton and Schubert (1954) recovered about 50 per cent of the total hexosamine of cartilage by repeated extraction with water during 92 days Partridge (1948) found that a soluble mucoid can be extracted from dried cartilage by a short treatment with water at 60—70 C Calcium chloride solutions have been used to isolate MPS from tissues (Meyer and Smyth 193 Meyer *et al* 1937) The yield on extraction with aline is low and the remaining polysaccharides can be extracted only with alkaline solutions (Blix and Snellman 1945 Einbinder and Schubert 1951)

Proteolytic enzymes

The introduction of enzymatic proteolysis of tissues as a means of liberating polysaccharides from protein has led to a most general method for the isolation of MPS Papain and pepsin tryp in have been used to liberate poly accharides by many investigators Papain lowers the vi co ity of chondroitin sulphate solutions (Muir 1956) but the molecular weight and size of chondroitin sulphate do not change on enzymatic digestion (Laurent 1957) The compositions of AMPS fractions liberated by papain pepsin trypsin or alkali did not differ but the yields were higher after enzymatic digestion (Szabo and Roboz Einstein 1962 Wood and Anastassiadis 1965 Meyer *et al* 1956)

Fractionation of mucopolysaccharides

When the MPS are extracted from tissue a mixture of polysaccharides is obtained The important and difficult step is to resolve this mixture into its components Several methods have been developed

Precipitation with ethanol

Meyer *et al* (1956) fractionated the AMPS of different ti ues by precipitating them from calcium acetate buffer by adding ethanol Other fractionation schemes using barium acetate instead of calcium acetate have been worked out by Jorpes and Gard ll (1948) as well as by Smith and Gallop (1953) In the majority of cases however, clear cut separation of MPS by the e methods is a tedious procedure and these procedures are diffi ult to u e when only small amounts of MPS are available Gardell (1957) modified the ethanol precipitation method by introducing a cellulose column

Column chromatography of cetylpyridinium complexes

The solubiliti s of quaternary ammonium complexes of polyanions in water are extremely low but the complexes are much more soluble in certain inorganic electrolyte solutions Scott (1960) showed that precipitates of different polyanions required different so-called critical alt concentrations before they dissolved The critical salt concentrations for hyaluronic acid chondroitin sulphates and heparin are very different

The method does not distinguish between the different chondroitin sulphates and heparitin ulphate As the charge den ity of the polymer increases the critical salt concentration increases too (Scott 1960) The exi tence of a close correlation between the molecular weight and the salt concentration necessary for the solubilization of cetylpyridinium complexes of keratosulphate and chondroitin sulphate was demonstrated by Laurent and Scott (1964)

It is difficult to recover finely divided precipitates from solutions The difficulty can be avoided by u ing column fractionation The first account of fractionation on a cellulose column appeared in a paper of Ro len (1956) The method was extensively investigated by Antonopoulos *et al* (1961, 1964), who developed also a micromethod employing gla s paper trips to fractionate mixtures containing 25—250 micrograms of each acid mucopolysaccharide

Chromatography and electrophoresis

Ringertz and Reichardt (1959 1960) u ed a cellulose ion exchange material (ECTEOLA) to fractionate AMPS Hyaluronic acid chondroitin sulphate and heparin had very different affinities for ECTEOLA which allowed their separation by elution with alt solutions of varying concentrations at an acid pH The recovery was nearly 100 % Berman (1962) isolated bovine vitreous hyaluronic acid from a diethylamino ethyl Sephadex column by elution with a linear concentration gradient of sodium chloride in a phosphate buffer S hmidt (1962) used DEAE Sephadex anion exchanger for the fractionation of AMPS A clear cut separation of hyaluronic acid, heparitin sulphate chondroitin ulphate and heparin was achieved Amounts of about 500 micrograms of AMPS could be resolved

Kerby (1953) used paper chromatography to separate heparin and chondroitin sulphate and different chondroitin sulphates can be eparated by the paper chromato graphic method of Castor and Dorstewitz (1964) Amounts of 10—20 micrograms of AMPS have been ucc ssfully eparated by chromatography on ion exchange paper with a odium chloride olution (Manley 1965)

Gardell *et al* (1950) found it possible to separate chondroitin sulphate and hyaluronic acid by electrophoresis in Hyflo Super Cel Rienits (1953) and Schultz Haudt (1957) studied the electrophoretic migration of AMPS in a phosphate buffer on filter paper trips Also many other buffers have been used in the separation of AMPS by paper electrophore is (Kerby 1955 Foster and Pearce 1961 Bollet 1958 Hall *et al* 1957 Mathews 1961) Toluidine blue and alcian blue are used to stain AMPS after electrophoresis Very small amounts of AMPS can be separated by electrophoresis on cellulo e acetate and the running time is much shorter than in paper electrophoresis The separation is good and the detection after ele trophoresis is satisfactory (Clausen and Ro enkast 196? Manley and Kent 1963 and Nanto 1963) Manl y (1965) and Friman and Brum h (1966) tried to estimate the relative con centrations of the different alcian blue taining components by photoscanning

Fractionation of AMPS by el ctrophoresis and by paper chromatography thus has som advantages over column fractionation for smaller amounts of MPS are required a more clear-cut paration is often obtained and the methods are less time consuming It has been difficult to characterize the fractions obtained by electro phoresis or by chromatography chemically It has however been possible to identify electrophoretically parated fractions of AMPS by gas chromatography (Lehtonen *et al* 1966)

Chemical analysis of the monosaccharides of mucopolysaccharides

Hydrolysis of polysaccharides

Hexosamines are very stable in acids and their liberation from tissue polysaccharides does not pose a serious problem Maximal yield of hexosamines from tissues are obtained by hydrolysis in 4 N hydrochloric acid for 15 hours at 100 C (Boas 1953) and the hexosamines are stable for as long as 24 hr in 4 N hydrochloric acid at 100 C (Good and Bessman 1964) A difficult problem is the quantitative analysis of glucuronic and iduronic acid because uronic acids released on acid hydrolysis of uronic acid containing material undergo partial decarboxylation (Link *et al* 1930) To overcome this difficulty Perry and Hulyalkar (1965) reduced the released uronic acids *in situ* to their acid stable aldonic acid derivatives by performing the hydrolysis of the polysaccharides in the presence of platinum and hydrogen Good results have been obtained when uronic acids have been liberated from polysaccharides by acid hydrolysis in 0 05 N hydrochloric acid in the presence of Dowex 50 (Anastassiadis and Common 1958 Dziewiatkowski 1962)

Gas liquid chromatography (GLC) of carbohydrates

The first report on the separation of sugars by gas liquid partition chromatography was that of McInnes *et al* (1958) Successful attempts to separate carbohydrates were made by converting them into completely methylated or acetylated derivatives These methods have been reviewed by Bishop (1962) The work of Sweeley *et al* (196) is a great advance in the analysis of sugars in biological materials They described the separation of almost 100 carbohydrates as their trimethylsilyl (TMS) derivatives by GLC The preparation of TMS derivatives of carbohydrates is a simple procedure the derivatives are rapidly and quantitatively formed in pyridine containing hexamethyldisilazane and trimethylchlorosilane at room temperature

Hexosamines

The determination of hexosamines is usually based on the Elson Morgan reaction (1933) Since that time many modifications have been proposed to increase the reliability and sensitivity of the method (Blix 1948, Boas 1953) Boas (1953) introduced a quantitative ion exchange method with Dowex 50 for the separation of hexosamines from interfering substances Exley (1957) and Gatt and Berman (1966) described methods applicable to 1—10 microgram quantities A method for the direct determination of N acetylhexosamines was developed by Morgan and Elson (1934) and modified by Aminoff *et al* (1952) and Reissig *et al* (1955) but the quantitative differentiation of glucosamine and galactosamine has not been possible using the methods of Elson and Morgan

For accurate quantitative determinations of glucosamine and galactosamine, it is necessary to separate them before the colour reactions Gardell (1953) described a method for the quantitative separation of glucosamine and galactosamine on Dowex 50 ion exchange resin using 0 3 N hydrochloric acid as eluent Comparatively large samples were needed for the analysis Pearson (1963) found that hexosamines were well

separated on elution from a column of Dowex 50 with citrate buffer of pH 5.0. For accurate determinations 30—100 micrograms of each compound was needed. Antonopoulos (1966) described a microprocedure for the quantitative separation of glucosamine and galactosamine; by this procedure amounts in the range of 1—25 micrograms can be analysed.

Analysis of hexosamines by GLC. According to Perry (1964) glucosamine and galactosamine can be analysed by gas chromatography of their N-acetylated trimethylsilyl derivative. Richey *et al.* (1964) obtained quantitative data for TMS ethers of N-acetylaminosugars. Karkkainen *et al.* (1965) described suitable conditions for the trimethylsilylation of unacetylated hexosamines as well as for gas chromatography of the TMS derivatives. The determination of glucosamine and galactosamine by this method is both simple and rapid.

Uronic acids

The carbazole method developed by Dische (1947) is the principal method for the determination of uronic acid. In quantitative determinations, however, the light absorption due to hexoses, which is about 5—7 % of that of uronic acids, and the interference of protein must be considered. Gregory (1960) and Bitter and Muir (1962) suggested the use of sodium tetraborate with sulphuric acid in the determination. All the chondroitin sulphates contain equimolar ratios of uronic acid to N-acetylhexosamine when the uronic acid is determined by the orcinol colour reaction, but the carbazole reaction gives markedly lower levels of iduronic acid (Khym and Doherty 1952). The presence of iduronic acid as a constituent of chondroitin sulphate B was reported by Hoffman *et al.* (1956) and Cifonelli *et al.* (1958) and uronic acid has been characterized as L-iduronic acid by Stoffyn and Jeanloz (1960). The orcinol reaction is also used for the determination of uronic acids but the results are not always satisfactory (Meyer *et al.* 1942, Dische 1955). Perry and Hulyalkar (1965) have analysed uronic acids by GLC and Lehtonen *et al.* (1966) used GLC for the detection of iduronic acid in MPS mixtures.

Neutral sugars

One of the colour reactions which has been used for the quantitative determination of hexoses is the anthrone reaction first described by Dreywood (1946). It has been modified by several investigators (Sattler 1948, Nikkilä and Pesola 1960). Dische (1955) compared the anthrone reaction with his modification of the cysteine reaction and drew the conclusion that both reactions have about the same sensitivity and specificity.

Many column chromatographic methods have been developed for the analysis of neutral sugars. Khym and Zill (1951) proposed an ion exchange column chromatographic method for the separation of neutral sugars in which the borate complexes of sugars are eluted from a strongly basic anion exchanger with borate buffers. A high degree of resolution and quantitation of neutral sugars was obtained when a boric acid-glycerol buffer was used (Walborg *et al.* 1965).

Analysis of neutral sugars by GLC. Richey *et al.* (1964) and Sawardeker and Sloneker (1965) have analysed monosaccharides quantitatively as their TMS derivatives using GLC. The method has been used for the analysis of sugars in many biological

samples serum and urine (Wells *et al* 1964) gastric MPS (Oates and Schrager 1965) tissues (Quan Ma and Wells 1965) glycosides and oligosaccharides (Wulff 1965) oligosaccharides, lipopolysaccharides glycolipids and gangliosides (Sweeley and Walker 1964) Difficulties are however often encountered in the analysis of sugar mixtures due to the great number of peaks arising from the different isomers of the monosaccharides To a certain extent resolution can be improved by appropriate choice of the stationary phase or by analysing sugar derivatives *e g* methylglycosides (Sweeley and Walker 1964) Bolton *et al* (1965) used methanolysis in their gas chromatographic investigations of glycoproteins Sawardeker *et al* (1965) reduced the monosaccharides quantitatively to their alditol derivatives with sodium borohydride This procedure eliminates the problem of multiple peaks since the alditols do not form isomers The data obtained by Sawardeker *et al* (1965) indicate that both the acetylation and reduction of monosaccharides are quantitative Morrison and Perry (1966) presented a procedure involving the oxidation of monosaccharides to the corresponding aldonic acids which could then be analysed by GLC A single derivative is obtained from each monosaccharide Adsorption chromatography of TMS ethers of sugars on silica affords a rapid method of fractionating a complex mixture of sugars before final analysis by gas liquid chromatography (Karkkainen *et al* 1966) Sugar mixtures can be quantitatively separated into amino sugars uronic acids and neutral sugars by this method

OBJECTS OF THE PRESENT INVESTIGATION

Acid mucopolysaccharides have possibly a role in fibrillogenesis and their part in the regeneration of tissues is generally admitted Because many changes have been observed to occur in the MPS in ageing connective tissues above all in cartilage, aortic tissue and skin the part played by MPS in ageing processes has engendered interest The sponge implantation method which was first employed by Boucel and Noble (1955) is a valuable technique for producing connective tissue Viscose cellulose sponge was later found to be a good implantation material for the production of reproducible samples of connective tissue (Viljanto and Kulonen 1962)

In the present study samples of connective tissues in various stages of development were produced by the sponge implantation technique The purpose was to study the MPS in these samples and thus obtain information about the ageing process of granulation tissue Newly developed methods, which permit specific determination of the monosaccharide components of the MPS and the characterization of microfractions of MPS, were necessary because of the complexity of the MPS mixtures The saline soluble and saline insoluble MPS were studied separately to obtain information about the participation of MPS in the formation of fibres

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Two groups of white Wistar rats were used as test animals The first group (A) comprised 139 rats and the second group (B) 126 rats The second experimental series was begun four months after the first series The weights of the rats on the day of implantation were 140—170 g and their ages 10—12 weeks The implants could easily be placed subcutaneously in these animals and the animals were able to produce new connective tissue Only female rats were used to avoid sex differences Prior to the implantation the rats were housed 10—30 to a cage (40×36×20 cm) The animals were fed standardized food which has been used in this laboratory for several years It was prepared daily and consisted of 100 parts of soybean 100 parts of corn 10 parts of defatted milk solids 5 parts of commercial margarine one part of sodium chloride and one part of calcium carbonate Once a week vegetables, cod liver oil yeast and boiled lung were added The dry mixture was mixed with water to a porridge The animals obtained water ad libitum from inverted bottles through metal tubes The diet was the same throughout the experiment After the implantation of the sponges the rats were kept in smaller cages (23×18×18 cm), two in each The temperature of the animal house was 20—25 C

IMPLANTATION OF SPONGES

Visella brand viscose cellulose sponge was used as implantation material The dry weights of the implanted cellulose pieces were 50—60 mg The implantation technique was that described by Viljanto (1964) Four pieces were implanted in each rat After periods of time varying from 1 to 10? days 10—20 rats were killed with ether and the four implants were removed from each rat The connective tissue capsule surrounding each piece was carefully removed and the pieces were placed immediately in a flask and stored frozen

CHEMICALS

Reagents

Alcian blue	Gurr & Co Ltd London England.
Anthrone	E Merck AG, Darmstadt, Germany
Benzene crystallizable, dried with calcium chloride and redistilled	E Merck AG
Carbazole	E Merck AG
Cetylpyridinium chloride	Recip AB Stockholm, Sweden
Dowex 2 (200—400 mesh)	Fluka AG, Chemische Fabrik Buchs SG, Switzerland
Dowex 50 (200—400 mesh)	Fluka AG

Ethyl acetate, for chromatography	E Merck AG
Gas Chrom P siliconized (100—120 mesh)	Applied Science Laboratories, State College, Pa, USA
Hexamethyldisilazane purum 98 %	Fluka AG
Hyaluronidase from bovine testes	Sigma Chemical Co, St Louis Missouri USA
N,N Dimethyl p aminobenzaldehyde	E Merck AG
Orcinol (guaranteed reagent)	E Merck AG
Oxoid(R) cellulose acetate sheets	Courtaulds Ltd Coventry England
Papain Crude Powder	Sigma Chemical Co
Pyridine reagent grade, redistilled anhydrous	J T Baker Chemical Co Phillisburg NJ USA
SE 30 methyl silicone polymer	General Electric Co Silicone Products Dept Waterford NY, USA
Sephadex A 25 medium	Pharmacia AB Uppsala Sweden
Silicic acid (Unisil, 200—300 mesh)	Clarkson Chemical Co Inc, Williamsport Pa, USA
Trimethylchlorosilane puris 99 %	Fluka AG
Whatman cellulose powder (ashless powder chemically prepared standard grade)	W & R Balston Ltd England

Reference compounds

Chondroitin sulphate B	Gift from Mr K von Berlepsch F Hoffman La Roche, Ltd, Basel Switzerland
Chondroitin sulphate C	Gift from Dr T Laurent Department of Medical Chemistry University of Uppsala Uppsala Sweden
Heparitin sulphate	Gift from Prof K Meyer Columbia University, College of Physicians and Surgeons, New York NY USA
Keratosulphate	Gift from Dr M B Mathews University of Chicago Chicago, Ill USA
N Acetylglucosamine	Sigma Chemical Co
N Acetylneuraminic acid	L Light & Co Ltd Colnbrook England
Chondroitin sulphate A	Mann Research Lab Inc New York, NY USA
L(—) Fucose	Fluka AG
D Galactosamine hydrochloride specific rotation +92 homogeneous in paper chromatography	Mann Research Lab
Galactose puris	E Merck AG
D Glucosamine hydrochloride specific rotation +72 8 homogeneous in paper chromatography	Mann Research Lab
D(+) Glucose analytical reagent grade	The British Drug Houses Ltd Poole Great Britain
D(+) Glucuronic acid lactone puriss	Fluka AG
D Mannose analytical grade	The British Drug Houses Ltd

EXTRACTION OF MUCOPOLYSACCHARIDES

Each specimen comprised 40—80 granulomas (in sponges) of one age. The wet weight of each specimen was determined and the specimens were homogenized in distilled water at +4 C in a Buhler rotating plate disintegrator. The homogenates were then frozen, freeze dried and weighed.

Each dry homogenate was suspended in 300 ml of 0.9 % sodium chloride solution and shaken at +4 C for 24 hr. The solution was centrifuged and the supernatant was collected. The extraction of the homogenate was repeated four times, after which no additional matter was removed.

The compounds soluble and insoluble in the 0.9 % salt solution were separately hydrolyzed with papain as described by Schiller *et al* (1961). Papain was activated in 0.1 M acetate buffer containing 1.0 M sodium chloride, 0.005 M cysteine and 0.005 M ethylenediaminetetraacetic acid for 30 minutes at 37 C. This activated papain was added in the ratio of 2 milligrams to each gram of dry homogenate to the reaction mixture, which was a 0.1 M acetate buffer (pH 5.5) containing 0.005 M cysteine and 0.005 M EDTA. The mixture was incubated at 63 C for 24 hrs. The same amount of papain was again added and the incubation was continued for 24 hours. The hydrolysate was deproteinized by adding trichloroacetic acid (TCA) to a final concentration of 10 per cent (w/v). After centrifugation the trichloroacetic acid was removed by shaking the mixture with several 300—400 ml portions of ether in a funnel. The MPS were precipitated at +4 C with 4 volumes of ethanol which contained 0.5 % sodium acetate and the precipitates were isolated by centrifugation and dissolved in distilled water to a concentration of about 1—2 %. The isolation scheme is presented in Fig. 1.

FRACTIONATION OF MUCOPOLYSACCHARIDES

Fractionation of mucopolysaccharides as their cetylpyridinium complexes on a cellulose column

Whatman cellulose powder was suspended in water and the fine particles were removed by decantation. The suspension was allowed to settle in a column (8×30 cm). The cellulose was successively washed with 0.5 N sodium hydroxide, 3 N sodium chloride, 0.1 N hydrochloric acid and distilled water. Fine particles were again removed by decantation. The air in the aqueous cellulose suspension was removed in an erlenmeyer flask by the suction of a water pump.

Cellulose columns (1.3 × 33 cm) were impregnated with a 1 % aqueous solution of *cetylpyridinium chloride (CPC). Chromatography of MPS (sample 5—10 mg)* was carried out stepwise as described by Antonopoulos *et al* (1961) with 1 % cetylpyridinium chloride solution and 0.[illegible], 0.[illegible]0, 1.00, 1.50 and 2.00 N magnesium chloride solutions. All the magnesium salt solutions contained 0.05 % cetylpyridinium chloride. A suitable flow rate was 4—8 ml/h. 1.3—[illegible] ml of polysaccharide solution was added to the top of column and was allowed to flow into the column. The column was then washed with 1 % cetylpyridinium chloride solution and eluted with the magnesium chloride solutions of increasing concentration using about two bed volumes of each.

Isolation of mucopolysaccharides from granulomas

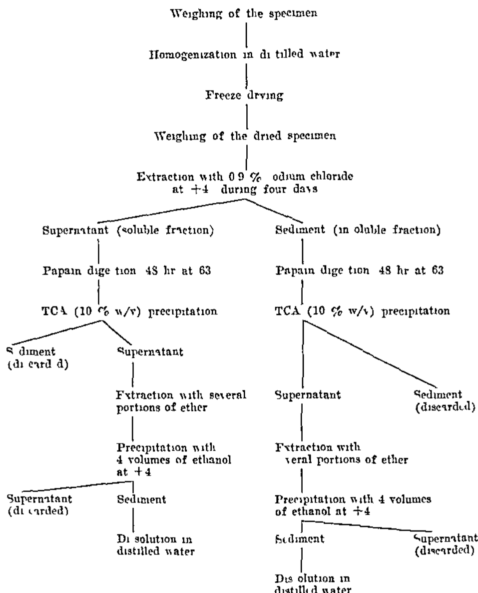


Fig 1

The MPS in the effluents were precipitated with four volumes of ethanol and the prec pitat w r li olve l in water To avoid variations in the separation of different ampl th alin soluble MPS from granulomas of different ages and the saline insolubl MPS w r fractionated simultaneously in six columns of the same size The elution l ut u ing the same flow rate

Fractionation of mucopolysaccharides on a DEAE Sephadex anion exchanger

This fractionation was done because it was hoped to gain a better resolution of the sulphated MPS than in the fractionation of the mucopolysaccharide-cetylpyridinium complexes This fractionation was performed as described by Schmidt (1962) as follows A column (2 × 30 cm) of Sephadex A 25 medium grade in the chloride form was used Before addition to the column the gel material was suspended in distilled water and allowed to swell, after which the fine particles were removed by decantation The swollen particles were added to a column fitted with a filter and washed with 0 5 N hydrochloric acid water, 0 5 N sodium hydroxide and water This procedure was repeated several times Finally the particles were washed with 0 5 N hydrochloric acid and water and then suspended in 0 1 N sodium chloride The columns were packed by allowing the slurry to settle Aqueous solutions of MPS samples containing 0 351—4 775 mg of hexosamine were allowed to flow into the column by gravity The polysaccharides were eluted with a continuous linear concentration gradient from water to a solution 2 0 N in sodium chloride and 0 02 N in hydrochloric acid (using a Phoenix Varipump, Model 3000 from Phoenix Precision Instrument Co Philadelphia Pennsylvania U S A) The fractionation was carried out at a constant flow rate of 55 ml/hour Fractions 6 5 ml in volume were collected The amount of uronic acid was determined in every second tube by the carbazole method (see below) and the chloride ion concentration was titrated with mercuric nitrate using diphenylcarbazone as indicator The polysaccharide fractions in the tubes were reprecipitated with four volumes of ethanol and the precipitates were isolated by centrifugation and dissolved in water

Electrophoretic fractionation of mucopolysaccharides

Electrophoresis of MPS was performed on Oxoid(R) cellulose acetate sheets A barbiturate buffer of pH 8 6 and ionic strength 0 125 (16 4 g diethylbarbituric acid 3 79 g sodium hydroxide and 12 95 g sodium acetate were dissolved in distilled water, 120 ml of 0 1 N hydrochloric acid was added and then distilled water to 2000 ml) was used as described by Näntö (1963) The buffer contained 100 ppm merthiolate and could be stored several months Strips of cellulose acetate were allowed to equilibrate in the buffer The amount of polysaccharides applied to each strip was 5—15 micro grams A constant voltage supply giving a potential gradient of 15 V/cm was used The running time was 40 minutes The buffer was replaced after 3 or 4 electrophoretic fractionations had been performed Immediately after the run the strips were passed through a 1 % solution of cetylpyridinium chloride and then washed with distilled water The strips were transferred to a 1 % (w/v) solution of Alcian blue in 25 % (w/v) acetic acid Staining was allowed to proceed several hours after which the strips were washed in 5 % acetic acid and dried between sheets of filter paper

QUANTITATIVE COLORIMETRIC ANALYSES

Hexosamines The polysaccharide samples were hydrolysed in 2 N hydrochloric acid at 103 C for 16 hours This time was found to give the best colour development in preliminary experiment

The hexosamines were freed from interfering chromogens by means of Dowex 50 cation exchange resin according to Boas (1953)

The colour reaction was performed by Blix s (1948) modification of the Elson Morgan (1933) method

Uronic acids These were determined by the carbazole method described by Bitt r and Muir (1962) and by the orcinol method as follows Four % orcinol in 1 5 % $FeCl_3$ was diluted 1 21 with 30 % hydrochloric acid One ml of sample was pipetted into a glass stoppered tube and 0 5 ml of distilled water and 4 5 ml of diluted orcinol reagent were added The tube was shaken, placed in boiling water for 0 minutes and cooled The colour intensity was measured with a Beckman B spectrophotometer at 6700 Å The standard curve was prepared from glucuronic acid

Hexoses Hexo es were analy ed by the anthrone method as modified by Nikkila and Pesola (1960)

N Acetylamino sugars The Morgan El on (1934) method was u ed in the modification of Reis ig *et al* (1955)

Sulphate Sulphate was determined by the benzidine method as modified by Antonopoulos (1962)

Sialic acid Hydrolysis of the samples and purification of the hydrolysates were carried out according to Zilliken and Whitehou e (1960) The colorimetric analysis was performed as described by Aminoff (1961)

Nitrogen The sample was digested by the Kjeldahl method

Nesslerization was carried out as described by Minari and Zilversmith (1963)

DETERMINATION OF SUGARS BY GAS CHROMATOGRAPHY

Gas chromatography

A Barb r—Colman M 10 chromatograph (Barber—Colman Co Wheelco Industrial Instruments Rockford Ill U S A) was u ed Conventional gas chromatographic practice as described by Wells *et al* (1964) was followed The conditions were the following column 1 % SE 30 on 100—400 mesh siliconized Gas Chrom P column temperature 145 C carrier gas nitrogen flow rate 30—50 cc/min inlet pressure 0 5 atm hydrogen flame ionization detector, anode voltage 200 V Amplification recorder full cale reading with a signal current of 6×10^{-9} amperes

Characterization and measurement of the peaks

Authentic monosaccharides were u ed separately or as internal standards in the gas chromatographic identifications The operating conditions were identical for the unknown sugars and for the reference sugars The relative retention time was calculated by determining the ratio of the retention time of a peak to that of sorbitol which was the internal standard

The peaks in th chromatograms were cut out with a calpel and weighed on a Mettler H 15 balan e (Nikkari 1965) Calibration coefficients were d termined for the peak areas of trim thyl ilyl ethers of different sugars Calibration curves were obtained

for each sugar by chromatographing different amounts of the sugar together with a constant amount of sorbitol and plotting the ratios of the total areas of the peaks of the sugar to the area of the peak of the standard against the ratios of the weights of the sugar to the weight of the standard The effect of sample size on the ratio was studied by injecting various amounts of sugars into the chromatograph All curves for the quantitative determinations were chromatographed with a recorder chart speed of 20 inches/hour

Hexosamines

Hydrolysis of mucopolysaccharides and purification of the hexosamines MPS were hydrolyzed in 2 N hydrochloric acid in sealed tubes at 100 C for 17 hr As natural MPS preparations often contain hexoses which may interfere in the gas chromatography of hexosamines, they have to be purified The separation of hexosamines from other sugars was carried out using Dowex 50 cation exchange resin (Boas 1953) The effluent was evaporated to dryness in an air stream on a water bath at 40—50 C The sugars were dissolved in a small volume of water and the solution was again evaporated to dryness

Trimethylsilylation Trimethylsilylation was carried out as described by Sweeley *et al* (1963) and by Karkkainen *et al* (1965) as follows The sample to be analysed was mixed with the internal standard sorbitol (10—100 micrograms in aqueous solution) in a conical centrifuge tube The solvent was evaporated in a stream of nitrogen Pyridine (0 175 ml) hexamethyldisilazane (0 05 ml) and trimethylchlorosilane (0 025 ml) were added successively immediately after drying the sample The mixture was shaken gently and allowed to stand at room temperature for 30 min after which the reaction mixture was evaporated to dryness in a nitrogen stream The trimethylsilyl ethers were extracted with 4—5 ml of redistilled n hexane by shaking vigorously or by using an ultrasonic vibrator The mixture was centrifuged the sediment was discarded and the clear supernatant which contained the trimethylsilyl ethers was concentrated to a suitable volume in a conical centrifuge tube with a stream of nitrogen

Gas chromatography The conditions for gas chromatography were described on page 26 0 5—2 0 microliters of each sample was injected into the gas chromatograph The calibration coefficients for the peak areas were determined from 10 replicate analyses

Neutral sugars

To find hydrolysis conditions giving the maximal yield of hexoses from tissue polysaccharides preliminary experiments were carried out using 0 5—2 0 N hydrochloric acid solutions and digestion periods varying from 2 to 24 hr at 100 C The best results were obtained when hydrolysis was carried out in 1 N hydrochloric acid at 100 C for 10 hr Degradation of neutral sugars could be almost completely avoided in these conditions and the ratios of galactose mannose and glucose were almost unchanged. Degradation of fucose was about 40 per cent in these conditions After hydrolysis the hexosamines were adsorbed on a Dowex 50 column The effluent which contained the hexoses was evaporated to dryness in a stream of air in a conical centrifuge tube kept in a water bath at 40—50 C The residue was dissolved in water and the water was again evaporated Trimethylsilylation and GLC were carried out as in the case of the hexosamines

Uronic acids

In preliminary experiments hydrolysis of polysaccharides was carried out in 1 N hydrochloric acid at 100°C for periods varying from 1 to 17 hr and in 0 05 N hydrochloric acid in the presence of Dowex 50 (H form) (Dziewiatkowski 1962) for 24 hr The highest recoveries were obtained when the polysaccharides were hydrolyzed in 1 N hydrochloric acid in sealed tubes at 100 C for three hours The hydrolyzate was evaporated to dryness in an air stream in a water bath at 40—50 C Trimethylsilylation, extraction with n hexane and gas chromatography were then performed as in the case of the hexosamines and hexoses

Analysis of the electrophoretically separated microfractions of mucopolysaccharides

Procedure After the electrophoresis of MPS on a cellulose acetate sheet, a narrow strip was cut from the sheet and stained with Alcian blue (1 % (w/v) solution in 25 % acetic acid) The identified bands on the unstained parts of the sheet were then cut out Each fraction in 8—16 bands was eluted with 3—5 ml of distilled water at room temperature for 6—8 hr For the determination of hexosamines the MPS fractions were hydrolysed in 1 5 N hydrochloric acid in sealed tubes at 103 C for 17 hr The separation and purification of the hexosamines were accomplished on columns of Dowex 50 Uronic acids and neutral sugars were analysed after hydrolysis of the fractions in 1 N hydrochloric acid at 100 C for 3 hr The amino sugars were removed from the hydrolyzates with Dowex 50 The hydrolyzates were then evaporated to dryness in an air stream Trimethylsilylation and extraction with n hexane were performed as described earlier The solution of extracted trimethylsilyl ethers was evaporated to a final volume of 10—20 microlitres in a conical centrifuge tube The cellulose acetate sheets were also eluted with water to determine the sugar released from the sheets

Column chromatography of trimethylsilyl (TMS) ethers of sugars

The trimethylsilyl ethers of sugars can be separated into classes by column chromatography on silica gel (Karkkainen *et al* 1966) Neutral sugars are separated from amino sugars and uronic acids on a silica gel column by eluting with benzene Elution with 1 % ethyl acetate in benzene separates uronic acid from amino sugars The organic solvents used in this method can be easily removed and the trimethylsilyl ethers can be injected into the gas chromatograph

Procedure The MPS were hydrolyzed and trimethylsilylation was carried out as described earlier The trimethylsilyl ethers were extracted with n hexane and the extract was concentrated in a stream of nitrogen Silica gel that had been dried at 150 C for 30 minutes and stored under dry hexane was dispersed in hexane and added with a Pasteur pipet to a column (90 × 4 mm) Trimethylsilyl ethers in a small volume of hexane were transferred onto the column Neutral sugars were eluted with benzene and amino sugars and uronic acids with ethyl acetate The effluents were evaporated to dryness in a nitrogen stream and the trimethylsilyl ethers were dissolved in hexane 1—2 microlitres of each hexane solution was pipetted into the gas chromatograph

DETERMINATION OF DIFFERENT CHONDROITIN SULPHATES

It is not possible to separate chondroitin sulphates A, B and C by column fractionation or electrophoresis. For the determination of chondroitin sulphate A and/or C, the method described by Mathews and Inouye (1961) was used. This method is based on the fact that N-acetylhexosamines liberated by hydrolysis with hyaluronidase behave normally in the Morgan—Elson reaction only when they are substituted in position 6 (chondroitin sulphate C), while substitution in position 4 (chondroitin sulphate A) completely suppresses colour formation.

Acetylhexosamine determinations after hyaluronidase hydrolysis were performed on all chondroitin sulphate fractions as described by Reissig *et al.* (1955).

For the detection of iduronic acid of chondroitin sulphate B, the uronic acids of the chondroitin sulphate fractions were determined as described on page 28.

OPTICAL ROTATION

Optical rotations were measured with a Perkin—Elmer 141 Polarimeter using the sodium line (5890 Å) at 25 C. All solutions measured were colourless and clear. Chondroitin sulphates A, B and C were used as reference compounds.

RESULTS

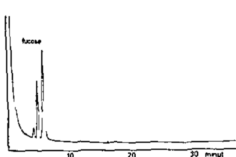
CHEMICAL ANALYSIS OF THE MUCOPOLYSACCHARIDES OF GRANULATION TISSUE

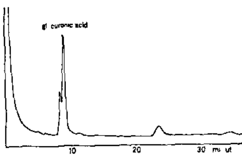
Separation of monosaccharides by gas chromatography

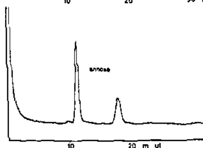
Gas chromatograms of trimethylsilylated sugars are presented in Fig 2 and Fig 3 On 1 % SE 30 at 145°C the trimethylsilyl ether of galactosamine produces only one peak while that of glucosamine produces two peaks The peaks of TMS ethers of amino sugars are well separated from one another The TMS ether of D galactose gives three peaks the TMS ethers of D mannose and D glucose give two each and the TMS ether of fucose three TMS ether of glucuronic acid lactone produces two peaks which are not completely separated The TMS ether of sorbitol which was used as internal standard gives one peak which is well separated from the peaks of the TMS ethers of the other sugars If hexoses and amino sugars are present in the same mixture the peaks obtained after silylation overlap in the gas chromatogram. The peaks due to the TMS ethers of β mannose and β galactose also overlapped but the relative area of the trimethylsilylated β mannose peak was small in comparison with the area of the trimethylsilylated α mannose peak when their equilibrium mixture in water was analysed

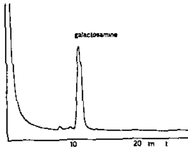
The separation of the TMS ether of iduronic acid obtained from chondroitin sulphate B and the TMS ether of glucuronic acid is seen in Fig 4 Iduronic acid produced only one peak on passage through 1 % SE 30 at 145°C

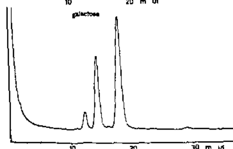
Separation of sugars into classes by column chromatography on silica gel and analysis by GLC Fig 5 shows the separation of TMS ethers of neutral sugars from TMS ethers of amino sugars and uronic acids on elution from a column of dried silica gel with benzene and ethyl acetate There were no signs of decomposition in the GLC tracing after column chromatography

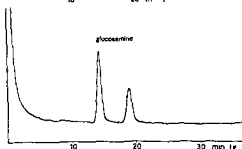


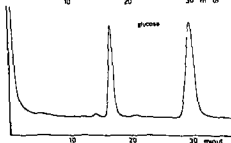












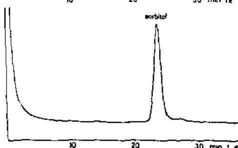


Fig 2 Gas chromatograms of trimethyl silyl derivatives of fucose mannose galactose and glucose 1% SE 30 column at 145

Fig 3 Gas chromatograms of trimethyl silylated glucuronic acid galactosamine glucosamine and sorbitol 1% SE 30 column at 145

Monosaccharide components of mucopolysaccharides

The monosaccharide components of MPS were analysed as described on pages 27—28 The hexosamines were always separated from other carbohydrates on columns of Dowex 50 as described by Boas (1953) Fewer extra peaks caused by impurities in the hydrolysates were present in the gas chromatograms of the trimethylsilylated neutral sugar fraction after than before column chromatography on silica gel The error of the quantitative method was determined by carrying out duplicate analyses on five samples of MPS The samples were divided into two equal portions after purification with Dowex 50 The relative standard deviation (calculated from duplicate determinations) was 4 8 % for galactosamine, 3 6 % for glucosamine, 6 8 % for galactose 5 0 % for glucose and 4 3 % for mannose Because the neutral sugars decomposed slightly in the acid hydrolysis, the total amounts of neutral sugars were calculated from the results of analysis by the anthrone method Two chromatograms for sugars are shown in Fig 6 and Fig 7

Because the peaks of trimethylsilyl ethers of β mannose and β galactose overlapped the area of the peak of trimethylsilyl ether of β mannose

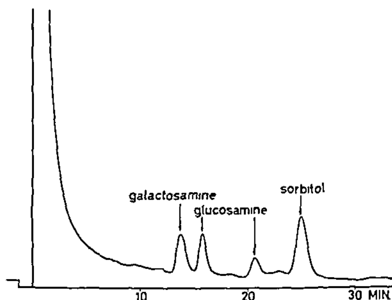


Fig 6 Gas chromatograms of trimethylsilylated ethers of the hexosamines in the saline soluble mucopolysaccharides of granulomas 15 days old 1 % SE 30 column at 140°

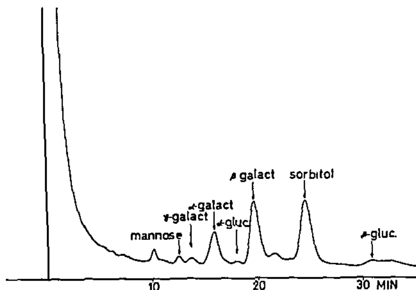


Fig " Gas chromatograms of trimethylsilylated ethers of neutral sugars liberated by hydrolysis from saline oluble mucopolysaccharides of granulomas 25 days old The ethers were prefractionated on a silica gel column and the fraction eluted with benzene from the column was analysed by GLC 1 % SE 30 column at 145

was calculated from the area of trimethylsilyl ether of α mannose (α mannose peak 71 1 %, β mannose peal 28 9 % of the total peal area of the trimethylsilylated mannose) The peak area of the trimethylsilyl ether of β galactose was calculated by subtracting the calculated area of the trimethylsilyl ether of β mannose from the peak area of the trimethylsilyl ether of β galactose (Fig 7)

THE FRACTIONATION OF MUCOPOLYSACCHARIDES

Separation of cetylpyridinium complexes of mucopoly saccharides on a cellulose column

Fractionation To achieve identical fractionations of all samples of MPS their cetylpyridinium complexes were fractionated as described on pages 23—24 Samples from granulomas of different ages were fractionated simultaneously on six columns The recovery of MPS calculated as hex o amine was a little over 80 % Solubility profiles for the cetylpyridinium MPS complexes are presented in Figs 8 and 9

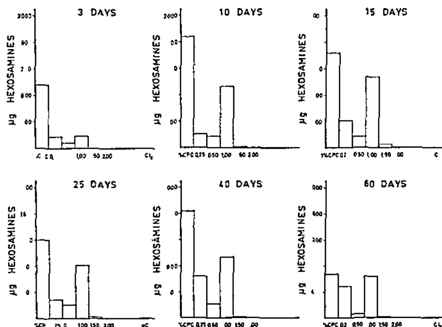


Fig 8 Solubility profiles for cetvlpyridinium complexes of saline oluble mucopolysaccharides of granulation ti sues of different ages

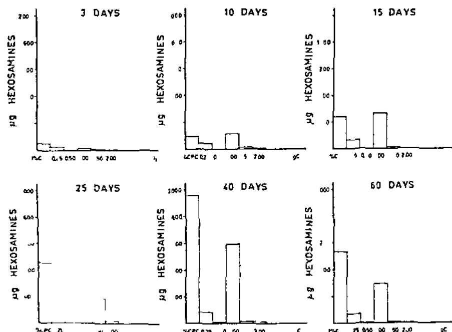


Fig 9 S lul ilit of cetvlpvri linium comj lexes of aline insoluble muco polvsac hari l f granulation tissue

Characterization of fractions The amounts of hexosamines uronic acids neutral sugars and sulphate in the fractions were determined by colorimetric methods and the molar ratios of the monosaccharides by gas chromatography The fractions were separated by electrophoresis on cellulose acetate In electrophoresis the fractions eluted with 0 50—2 00 N magnesium chloride moved at the same rate as chondroitin sulphate The fraction eluted with 0 25 N magnesium chloride was divided on electrophoresis into two fractions one of which moved like hyaluronic acid and other remained nearer the cathode this latter fraction was also present in the cetylpyridinium chloride eluant A fraction with a mobility equal to the mobilities of keratosulphate and heparitin sulphate was present among the MPS eluted with an aqueous cetylpyridinium chloride solution Figs 10—14 show the separation of monosaccharides obtained after hydrolysis of the MPS fractions The fraction eluted by cetylpyridinium chloride contained both glucosamine and galactosamine and a large amount

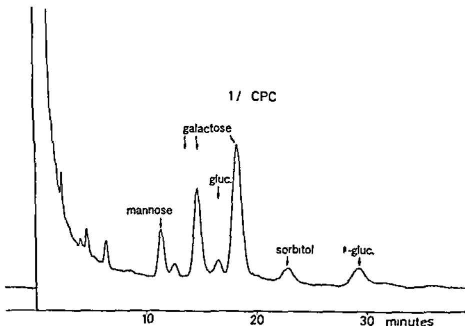


Fig 10

Figs 10—14 Gas chromatogram of trimethylsilylated amino sugars and neutral sugars liberated by hydrolysis from fractions of saline soluble polysaccharides of 60 day old granulomas eluted from cellulose columns The trimethylsilyl ethers of the neutral sugars derived from the polysaccharides of the fraction eluted with 1% cetylpyridinium chloride were purified on a silica gel column before gas chromatography on a 1% SE 30 column at 143 The peaks of β mannose and β galactose overlap

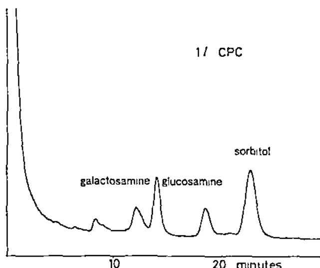


Fig 11

of neutral sugars Because this fraction was electrophoretically divided into two components it can be supposed that heparitosulphate (or heparitin sulphate) and a polysaccharide from glycoprotein or a neutral mucopolysaccharide were present in this fraction The uronic acid content was low The fraction eluted by 0 25 N magnesium chloride contained small amounts of galactosamine and neutral sugars in addition to glucosamine These

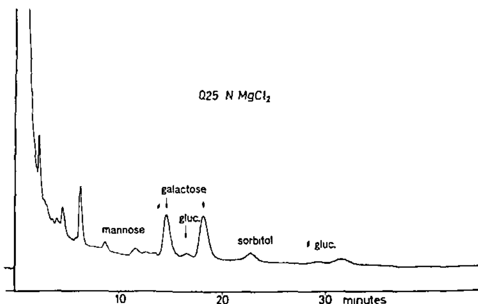
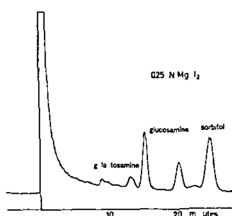


Fig 12



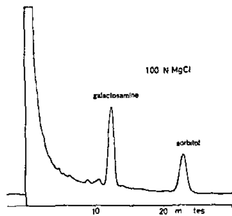


Fig 13 *Fig 14*

results show that the polysaccharide which was present in the cetyl pyridinium chloride effluent was present in addition to hyaluronic acid in this fraction Galactosamine was the only sugar detected by gas chromatography of the fractions eluted by 0 50—2 00 N magnesium chloride solutions after their hydrolysis in 2 N hydrochloric acid for 17 hrs at 100°C Thus chondroitin sulphate was completely separated from the other polysaccharides by fractionation of the cetylpyridinium complexes of MPS

Chromatography on DEAE Sephadex anion exchange resin

Fractionation

A column (2×20 cm) of Sephadex A 25 medium grade was used The polysaccharides were eluted with a continuous linear salt gradient from water to a 2 0 N sodium chloride solution 0 02 N in hydrochloric acid at a constant flow rate using a gradient pump Every sample of soluble and insoluble polysaccharides was divided into three or four fractions The recovery of hexosamine containing material was only a little over 70 per cent To achieve similar fractionation of all samples the same column the same gradient and the same flow rate were used Fractions 6 5 ml in volume were collected Some differences were however observed in the separation of fractions of different samples The cause of this may have been that the pressure compressed the Sephadex column during chromatography A typical elution pattern is shown in Fig 15

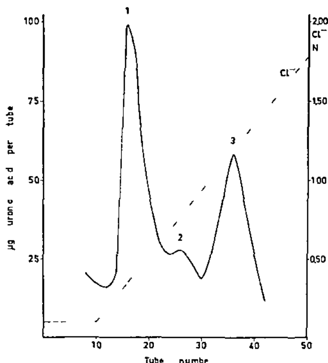


Fig 15 Elution pattern of saline insoluble mucopolysaccharides in 10 day old granuloma Elution was performed with a continuous linear gradient from water to 2 0 N sodium chloride at a constant flow rate of 35 ml/hr Fractions 6 5 ml in volume were collected Numbers 1 2 and 3 refer to fractions pooled for later analysis

Characterization of fractions

Electrophoresis and analysis of the components of polysaccharides in the fractions were carried out as after cetylpyridinium chloride fractionation Gas chromatograms of the monosaccharides in the fractions 1 and 2 are shown in Figs 16—19 Galactosamine was the only sugar detected by gas chromatography in the fraction 3 after hydrolysis in 2 N hydrochloric acid for 17 hrs at 100°C and this fraction had a mobility equal to that of chondroitin sulphate in electrophoresis Fractions 1 and 2 contained neutral sugars amino sugars and uronic acids The uronic acid content was much higher in fraction 1 than in fraction 2 Fraction 1 was divided on electrophoresis into three components the first moved like kerato

lphate and heparitin sulphate the second like hyaluronic acid and the ird remained nearer the cathode Electrophoresis divided fraction 2 to two components one of which moved like keratosulphate and heparitin lphate and other remained nearer the cathode like hyaluronic acid

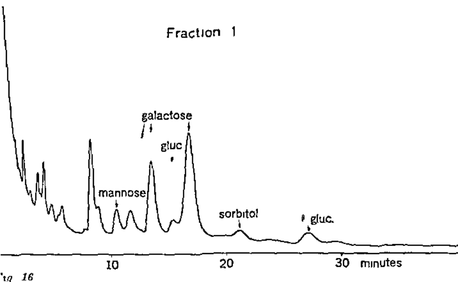


Fig 16

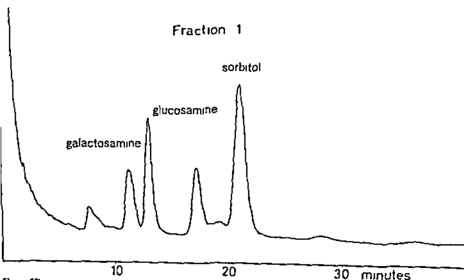


Fig 17

Figs 16—19 Gas chromatograms of trimethylsilyl ethers of amino sugars and neutral sugars liberated by hydrolysis from polysaccharide fractions isolated by DEAE Sephadex fractionation from the saline soluble mucopolysaccharides in 10 day old

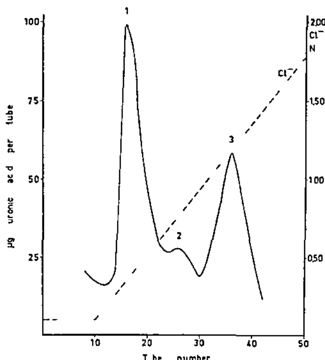


Fig 15 Elution pattern of saline in oluble mucopoly accharides in 10 day old granulomas Elution was per formed with a continuous linear gradient from water to 2 0 N sodium chloride at a constant flow rate of 55 ml/hr Fractions 6 5 ml in volume were collecte l Numbers 1 2 and 3 refer to fraction pooled for later analysis

Characterization of fractions

Electrophoresis and analysis of the components of polysaccharides in the fractions were carried out as after cetylpyridinium chloride fraction ation Gas chromatograms of the monosaccharides in the fractions 1 and 2 are shown in Figs 16—19 Galactosamine was the only sugar detected by gas chromatography in the fraction 3 after hydrolysis in 2 N hydro chloric acid for 17 hrs at 100°C and this fraction had a mobility equal to that of chondroitin sulphate in electrophoresis Fractions 1 and 2 contained neutral sugars amino sugars and uronic acids The uronic acid content was much higher in fraction 1 than in fraction 2 Fraction 1 was divided on electrophoresis into three components the first moved like kerato-

Separation by electrophoresis

Fractionation

Both the saline soluble and the saline insoluble MPS of granulation tissue were separated electrophoretically into four fractions (Figs 20—21) The electrophoretic mobility of fraction D was equal to that of chondroitin sulphate Fraction C moved at the same rate as the reference samples of heparitin sulphate and keratosulphate Fraction B had a mobility similar to that of hyaluronic acid which had been isolated from human umbilical cord and fraction A moved the shortest distance towards the anode

Characterization of fractions

Gas chromatograms of the trimethylsilyl ethers of monosaccharides in the electrophoretically separated fractions are shown in Figs 22—25

The results of the gas chromatographic analyses of the electrophoretically separated fractions are presented in Table I A small amount of glucosamine (probably an impurity) was present in fraction D of saline soluble MPS The quantity of sugars in fraction A of the saline soluble MPS was very small so that the possible presence of mannose could not be confirmed in the gas chromatogram When pure cellulose acetate sheets were eluted with water some glucose was observed in the effluent it was present also in all the MPS fractions

On the basis of this semiquantitative determination of the carbohydrate components fraction A contained polysaccharides from glycoproteins or neutral mucopolysaccharides fraction B was hyaluronic acid fraction C resembled keratosulphate and fraction D chondroitin sulphate Because fraction D did not contain iduronic acid chondroitin sulphate B was not present in the fraction Glycoproteins or neutral mucopolysaccharides and keratosulphate could not be separated or separately identified by fractionation with cetylpyridinium chloride or on DEAE Sephadex and thus the possible presence of keratosulphate could be positively established only by electrophoresis

The quantity of sugars in samples pooled from 6—12 electrophoretic sheets (total MPS about 60—120 microg) was large enough for their identification After hydrolysis for 3 hr (for the determination of uronic acids and neutral sugars) small unidentified peaks possibly due to impurities were observed in the gas chromatograms

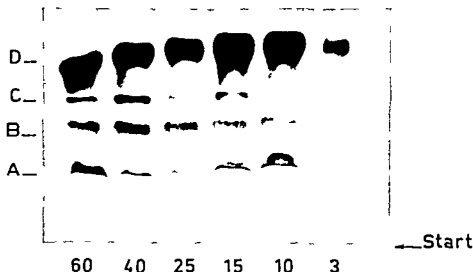


Fig 20 Electrophoretic patterns of saline soluble mucopolysaccharides from granulomas of different age Barbiturate buffer of pH 8 6, anode voltage 100 V, and running time 40 min

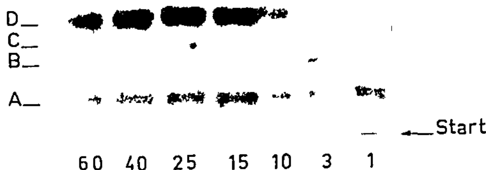


Fig 21 Electrophoretic patterns of saline insoluble mucopolysaccharides from granulomas of different age Barbiturate buffer of pH 8 6, anode voltage 100 V, and running time 40 min

Fig 22. Gas chromatogram of trimethyl silyl eth rs of amino sugars of ele tro phoretically eparated fractions of aline soluble mucopoly accharides from 25-day old granulomas Fractions A—D are tho e indicated in Fig 21 The samples were obtained from 6 electrophoresis strips containing about 60 micrograms of total mucopoly accharides 1 % SE 30 column at 145

Fig 23 Gas chromatogram of trimethyl silyl ethers of uronic acids and neutral sugars liberated from electrophoretically separated fractions of saline soluble muco polysaccharides from 25 day old granulomas Fractions A—D are those indicated in Fig 21 The samples were obtained by eluting 12 electrophoresis strips containing about 120 micrograms of total mucopolysaccharides 1 % SE 30 column at 145

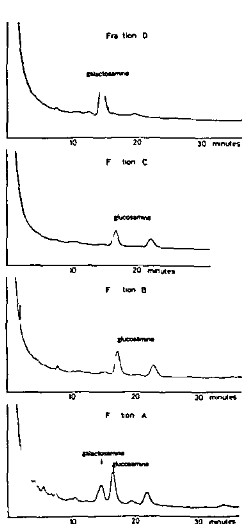


Fig 24 Cas chromatogram of trimethyl silyl ethers of amino ugars liberated from electrophoretically eparated fra tions of saline-in oluble mucopolysaccha rides from 25 day old granuloma Fractions A—D are those indicated in Fig 2. The amples were obtained by eluting 6 elec trophoresis strips containing about 60 micrograms of total mucopoly accharide 1 % SE 30 column at 145

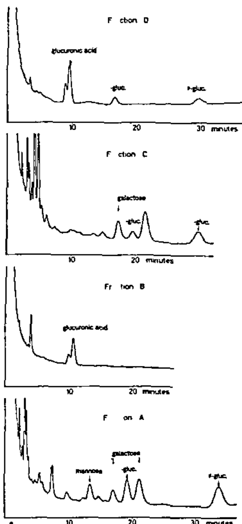


Fig 25 Gas chromatogram of trimethyl silyl ethers of uronic acids and neutral ugars liberated from electrophoretically eparated fractions of saline insoluble mucopoly accharides from 25 day old gran uloma Fractions A—D are those indi cated in Fig 20 The amples were ob tained by eluting 10 electrophore is strips containing about 100 microgram of total mu opoly acchari les 1 % SF 30 column at 145

Table I

Fraction	Glucosamine	Galactosamine	Glucuronic acid	Galactose	Mannose
Soluble					
A	+	+		+	
B	+		+		
C	+			+	
D	(+)	+	+		
Insoluble					
A	+	+		+	+
B	+		+		
C	+			+	
D		+	+		

Determination of different chondroitin sulphates

As already mentioned it is not possible to separate chondroitin sulphates A B and C by column fractionation or electrophoresis Because iduronic acid was not found in the MPS extracted from granulation tissue or in chondroitin sulphate fractions obtained by column fractionation (Fig 26) or electrophoresis it cannot be concluded that chondroitin sulphate B is present in granulation tissue

Acetylhexosamine determinations were performed after hyaluronidase hydrolysis on all chondroitin sulphate fractions obtained by column fractionation on cellulose and DEAE Sephadex The results are shown in Table II According to these results the amount of chondroitin sulphate C was very small if it was present at all This amount did not vary significantly with tissue age The optical rotations of the chondroitin sulphate fractions confirm the prevalence of chondroitin sulphate A (Table III) The carbazole method gives a markedly lower iduronic acid content than the orcinol method the ratio of the two contents is about 0 5 Therefore the uronic acid contents of chondroitin sulphate fractions were determined using both the carbazole and the orcinol methods The results are shown in Table IV The ratio of the results obtained with these methods was close to unity only for MPS from granulomas 10 days old the ratio for MPS

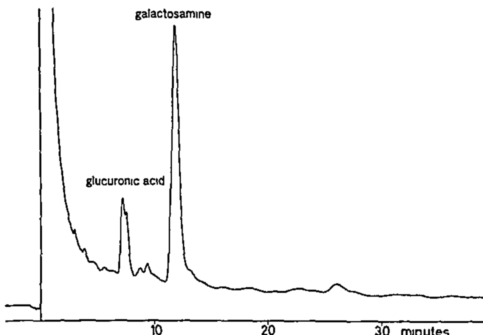


Fig 26 Gas chromatograms of trimethylsilyl ethers of sugars in the chondroitin sulphate fraction isolated by cellulose column fractionation of saline insoluble mucopolysaccharides from 25 day old granulomas 1% SE 30 column at 145 The mucopolysaccharides were hydrolysed in 1 N hydrochloric acid for 3 hours at 100

from older granulomas was low (about 0 5) The molar ratio of uronic acid to hexosamine as measured by the orcinol method was too high this was probably due to nonspecific reactions of orcinol

Table II Percentages of acetylhexosamines from hexosamines in chondroitin sulphate fractions obtained with cetylpyridinium chloride on cellulose and with magnesium chloride on DEAE Sephadex

The acetylhexosamines were determined by the Morgan—Elson method after hydrolysis of the chondroitin sulphates with hyaluronidase

Age of granulomas	Fractionation on cellulose		Fractionation on DEAE Sephadex	
	Soluble	Insoluble	Soluble	Insoluble
10 days	8 2	4 4	1 2	1 0
15	4 9	4 5	3 9	6 4
25	[illegible]	4 4	—	—
40	[illegible]	4 2	4 2	1 8
60	[illegible]	4 8	3 8	4 4

Table III Optical rotations of chondroitin sulphate fractions obtained by chromatography on DEAE Sephadex

The optical rotations were measured with a Perkin—Elmer 141 Polarimeter using the sodium line (5890 Å) at +25 C

Age of granuloma in days	Saline soluble chondroitin sulphate α_D^{25}	Saline insoluble chondroitin sulphate α_D^{25}
10	—33.7	—38.5
40	—31.8	—35.2
60	—35.0	—30.5

Table IV The ratios of uronic acid contents of chondroitin sulphate fractions obtained with cetylpyridinium chloride on cellulose or with magnesium chloride on DEAE Sephadex as determined by the carbazole and orcinol methods

Age of granuloma in days	Ratios of uronic acid contents as determined by the carbazole and orcinol methods	
	Soluble chondroitin sulphate	Insoluble chondroitin sulphate
10	0.93	0.84
15	0.43	0.44
25	0.44	0.52
40	0.53	—
60	0.50	0.63

THE EFFECT OF AGE OF GRANULATION TISSUE

Growth of granulation tissue

Fig 27 shows the wet and Fig 28 the dry weights of granulomas of different ages The results were obtained by weighing the pieces of each specimen simultaneously and dividing the value by the number of pieces The weights of the granulomas without sponge were obtained by subtracting the mean weight 56.2 mg of the cellulose sponge from the mean weight of one piece The number of pieces in each specimen was 38—80

The wet weights of the granuloma tissues of series A rose to a maximum on the 15th day and the wet weights of the granuloma tissues of series B on the 10th day Subsequently the wet weights of the granulomas fell

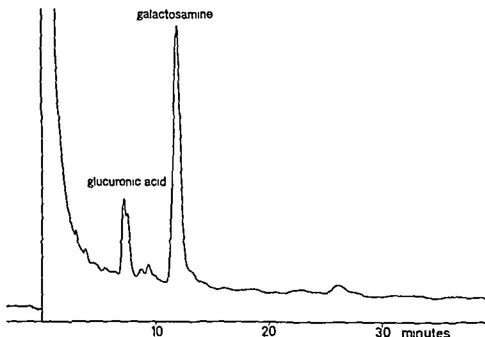


Fig 26 Gas chromatogram of trimethylsilyl ethers of sugars in the chondroitin sulphate fraction isolated by cellulose column fractionation of saline insoluble mucopolysaccharides from 25 day old granulomas 1% SE 30 column at 145 The mucopolysaccharides were hydrolysed in 1 N hydrochloric acid for 3 hours at 100

from older granulomas was low (about 0 5) The molar ratio of uronic acid to hexosamine as measured by the orcinol method was too high this was probably due to nonspecific reactions of orcinol

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The acetylhexosamines were determined by the Morgan—Elson method after hydrolysis of the chondroitin sulphates with hyaluronidase

Age of granulomas	Fractionation on cellulose		Fractionation on DEAE Sephadex	
	Soluble	Insoluble	Soluble	Insoluble
10 days	8 2	4 4	1 2	1 0
15	4 9	4 5	3 9	6 4
[illegible]	[illegible]	4 4	—	—
40	[illegible] 4	4 2	4 2	1 8
60	[illegible]	4 8	3 8	4 4

Table III Optical rotations of chondroitin sulphate fractions obtained by chromatography on DEAE Sephadex

The optical rotations were measured with a Perkin—Elmer 141 Polarimeter using the sodium line (5890 A) at +25 C

Age of granuloma in days	Saline soluble chondroitin sulphate α_D	Saline insoluble chondroitin sulphate α_D^{25}
10	—33 7	—38 5
40	—31 8	—35 2
60	—35 0	—30 5

Table IV The ratios of uronic acid contents of chondroitin sulphate fractions obtained with cetylpyridinium chloride on cellulose or with magnesium chloride on DEAE Sephadex as determined by the carbazole and orcinol methods

Age of granuloma in days	Ratios of uronic acid contents as determined by the carbazole and orcinol methods	
	Soluble chondroitin sulphate	Insoluble chondroitin sulphate
10	0 93	0 84
15	0 43	0 44
25	0 44	0 52
40	0 53	—
60	0 50	0 63

THE EFFECT OF AGE OF GRANULATION TISSUE

Growth of granulation tissue

Fig 27 shows the wet and Fig 28 the dry weights of granulomas of different ages The results were obtained by weighing the pieces of each specimen simultaneously and dividing the value by the number of pieces The weights of the granulomas without sponge were obtained by subtracting the mean weight 56 2 mg of the cellulose sponge from the mean weight of one piece The number of pieces in each specimen was 38—80

The wet weights of the granuloma tissues of series A rose to a maximum on the 15th day and the wet weights of the granuloma tissues of series B on the 10th day Subsequently the wet weights of the granulomas fell

Series A
Series B
Mean

1000
750
500
250
mg / piece
30
60
90
120 DAYS

Fig 27 Wet weights of granulation tissues of different ages The weights were obtained by subtracting the mean weight 56 2 mg of the cellulose sponge from the mean weights of the sponge after their removal from the animals

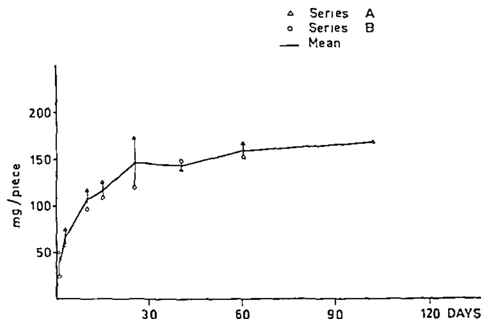


Fig 28 Dry weights of granulation tissues of different ages The weights were obtained by subtracting the mean weight of the cellulose sponge (56 2 mg) from the mean dry weight of the pieces

steadily and were smaller on the 102nd day (the last day when specimens were removed) than on the 1st day The dry weight of the tissue increased up to the 25th day after implantation in series A and up to the 40th day in series B The variations in the tissue dry weights were small during the period from the 25th to the 102nd day The water content of the tissue was highest, 89 0 per cent of the wet weight of the tissue, on the 1st day and lowest 74 4 per cent, on the 102nd day Because the dry weights were determined after freeze drying the weights are somewhat higher than they would have been after effective drying by heating

Saline soluble mucopolysaccharides

Colorimetric analyses The hexosamine uronic acid, neutral sugar sulphate and nitrogen contents of the isolated saline soluble MPS are shown in Fig 29 The MPS of series A and series B were analysed separately The hexosamine uronic acid and sulphate contents increased during the first 15—25 days and varied slightly later The mean contents of hexosamine

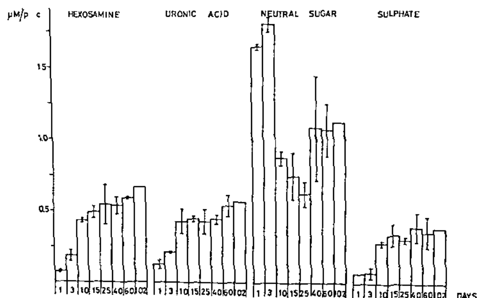


Fig 29 The components of the saline soluble mucopolysaccharides in granulation tissues of different ages The amounts in series A and series B were determined separately The heights of the columns give the mean amounts of the two series µM = µmole

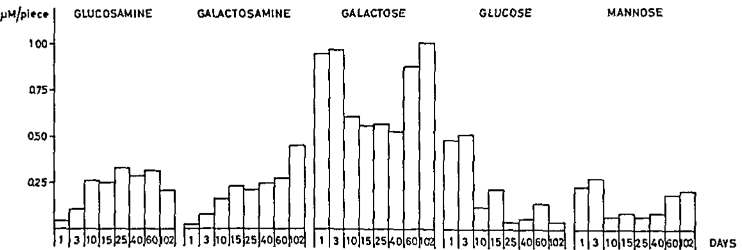


Fig 30 Amino sugars and neutral sugars of saline soluble mucopolysaccharides from granulomas The total amounts of hexosamines and neutral sugars were calculated from the results of colorimetric analyses (Fig 29) and the molar ratios of the monosaccharides were determined by gas chromatography

uronic acid and sulphate were higher on the 60th day than on the 25th day and the analyses of MPS from granulomas removed 102 days after implantation in series A gave contents of hexosamine and uronic acid that were a little higher than the mean contents on the 60th day The content of neutral sugars was highest on the first three days The nitrogen content varied from 0 379 to 3 236 µmoles per piece, but was not correlated with the age of the granuloma this shows that variable amounts of protein were present in the MPS extracts The content of sialic acid was also determined it was 2 6 µg/piece on the first day and 1 2 µg/piece on the third day Sialic acid was not detected in older granulomas

Gas chromatography The molar ratios of amino and neutral sugars in total MPS of granulation tissues of different ages are shown in Fig 30 The glucosamine content increased rapidly during the first ten days, but then varied only slightly The galactosamine content increased less rapidly and was highest in granulomas 102 days old The contents of galactose, glucose and mannose were high on the first three days but then decreased rapidly The contents of galactose and mannose were again higher in the oldest granulomas

The fractions of mucopolysaccharides obtained by elution of their cetyl pyridinium complexes from cellulose

Fig 31 shows the amounts of the MPS fractions (expressed as the amounts of hexosamines in the fractions) obtained on fractionation of their cetylpyridinium complexes Chondroitin sulphate was well separated from the other MPS Hyaluronic acid was not completely separated from glycoproteins or neutral mucopolysaccharides but the amount of hyaluronic acid could be calculated from the amount of uronic acid found in this fraction A keratosulphate like polysaccharide and glycoproteins or neutral mucopolysaccharides were not separated from each other The content of hyaluronic acid increased with the age of the granuloma but was relatively low The content of chondroitin sulphate increased up to the 25th day but varied very little later The fraction containing polysaccharide resembling keratosulphate and glycoproteins or neutral mucopolysaccharides was the largest of the soluble MPS fractions its content increased at first but varied little later The amount of the fraction was calculated from the amount of hexosamine and was much smaller than the amount calculated from the amount of neutral sugars

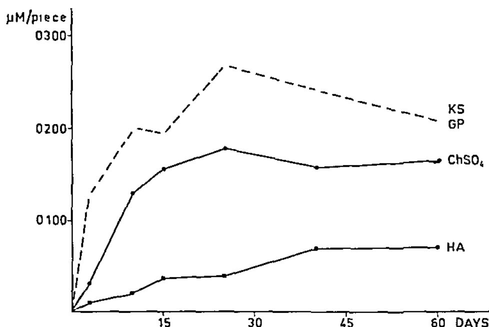


Fig 31 Amounts of hexosamines in the fractions obtained on fractionation of c tylpyridinium complexes of aline soluble mucopoly accharides on a cellulose column HA = hyaluronic aci l ChSO = chondroitin sulphate KS = polysaccharide re emi ling keratosulphate GP = glycoprotein or neutral mucopolysaccharide

The fractions of mucopolysaccharides obtained by chromatography on DE AF Sephadex

Chondroitin sulphate was well separated from the other MPS also by this method Hyaluronic acid was not separated from the fraction containing neutral sugars The polysaccharide resembling keratosulphate and glyco proteins or neutral mucopolysaccharides were eluted togethei The hyal uronic acid content was determined from the amount of uronic acid in frac tion 1 (Fig 15) However the neutral sugars of glycoproteins may have interfered in the carbazole reaction Electrophoresis revealed the presence of small amounts of hyaluronic acid in some samples of fraction 2 (Fig 15), but these amounts could not be quantitatively determined

Fig 32 shows the amounts of different components obtained by column chromatography on DEAE Sephadex

Also the results obtained by this method ... that the hyaluronic acid content increases with the age of ... gran ... that the chondroitin sulphate content increases ... then remains

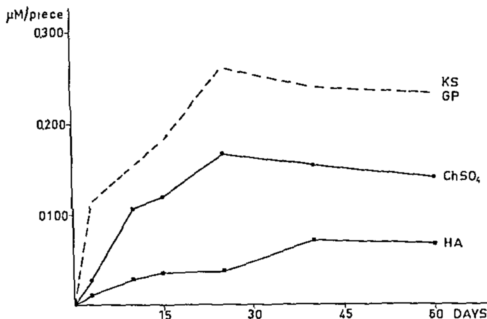


Fig 32 Amounts of hexoamines in fractions obtained on column chromatography of saline soluble mucopolysaccharides on DEAE Sephadex Abbreviations as in Fig 31

approximately constant The amount of the fraction containing neutral sugars expressed as numbers of moles of hexosamine was high already on the third day rose to a maximum on the 25th day and decreased slightly later Some differences are seen in the results of these two procedures but both groups of results show the same age variation

Saline insoluble mucopolysaccharides

Colorimetric analyses The hexosamine uronic acid, neutral sugar sulphate and nitrogen contents in the saline insoluble MPS of granuloma tissues of series A and B were determined similarly as the contents in the saline soluble MPS (Fig 33) The contents of hexosamine uronic acid and sulphate increased steadily up to the 40th day after which the increases were small The content of neutral sugars also reached a maximum on the 40th day but decreased later The content of nitrogen in the MPS preparations was about 10 per cent and was higher on average in series B than in series A although same method was used to extract the MPS The

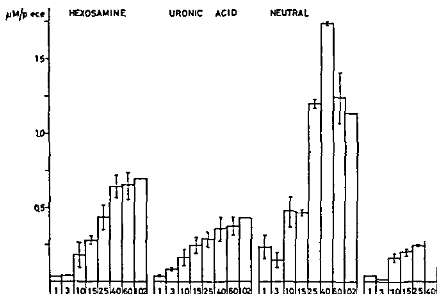


Fig 33 Components of the saline insoluble mucopolysaccharides of gra[illegible] tissues of different ages The amounts in series A and series B were determin[illegible] rately The heights of the columns give the mean amounts in these two series

content of sialic acid was 1 3 µg/piece on the first and 0 4 µg/piec[illegible] the third day but the acid was not found in later samples

Gas chromatography The molar ratios of amino sugars and galactos[illegible] in the saline insoluble MPS are shown in Fig 34 Both the glucosamine and galactosamine contents increased up to the 40th day, but then remained approximately constant The content of galactose was high in older granulomas The contents of glucose and mannose could be determined in only a few samples no significant increase with age was found The greatest amount of mannose (0 22 µmole/piece) and the greatest amount of glucose (0 13 µmole/piece) were present in the granulomas 40 days old

The fractions of mucopolysaccharides obtained by elution of their cetyl pyridinium complexes from cellulose

The saline insoluble MPS were separated in the same way as the soluble MPS Fig 35 presents the changes in the MPS fractions with age The content of hyaluronic acid was low in saline insoluble MPS and significant changes with age were not observed A low content of chondroitin sulphate was found already on the third day and the content increased up to the 40th day The fraction containing keratosulphate like polysaccharide and

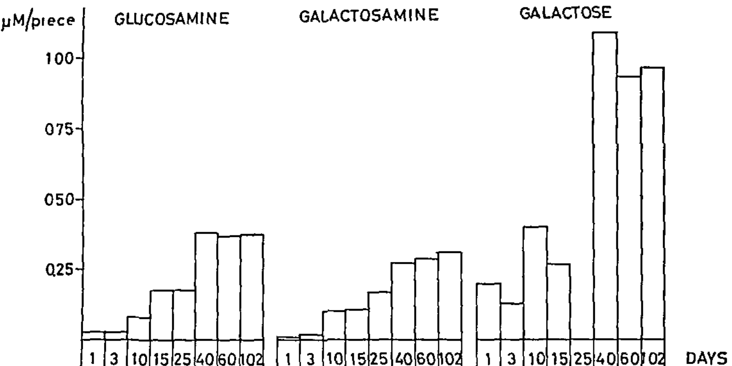


Fig 34 Amino sugars and neutral sugars in saline insoluble mucopolysaccharides of granulomas The total amounts were calculated from the results of colorimetric analyses (Fig 33) and the molar ratios of the monosaccharides were determined by gas chromatography

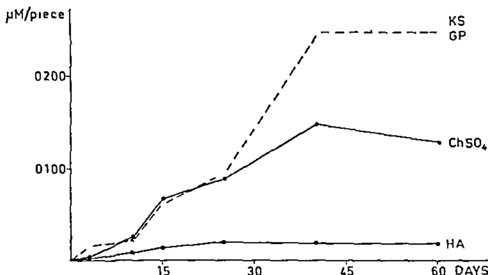


Fig 35 Amounts of hexosamine in the fractions obtained on fractionation of cetyl pyridinium complexes of saline insoluble mucopolysaccharides on a cellulose column Abbreviations as in Fig 31

polysaccharides from glycoproteins or neutral mucopolysaccharides was small during the first 10 days increased steadily up to the 25th day and subsequently more rapidly up to the 40th day

The fractions of mucopolysaccharides obtained by chromatography on DEAE Sephadex

Fig 36 presents the results obtained by this method These agree well with those obtained by cetylpyridinium chloride fractionation The content

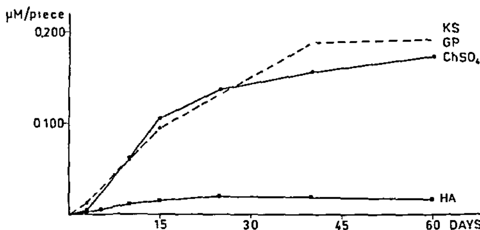


Fig 36 Amounts of hexosamines in the fractions obtained on column chromatography of saline insoluble mucopolysaccharides on DEAE Sephadex Abbreviations as in Fig 31

of chondroitin sulphate increased up to the 60th day The fraction containing keratosulphate like polysaccharide and glycoproteins predominated in the oldest granulomas but its content was somewhat lower than the content obtained by cetylpyridinium chloride fractionation This may be due to the relatively low recovery in the DEAE Sephadex fractionation

Comparison of the changes in soluble and insoluble mucopolysaccharides with age

The proportions of hexosamines in saline soluble and saline insoluble MPS are shown in Fig 37 The hexosamine content increased rapidly in both fractions but the increase began earlier in the soluble fraction The increase was rapid to the 25th day in the soluble and up to the 40th day in the insoluble fraction After the 40th day a small increase was observed in both fractions and the content of hexosamine in the insoluble MPS was a little higher than content of hexosamine in the soluble MPS

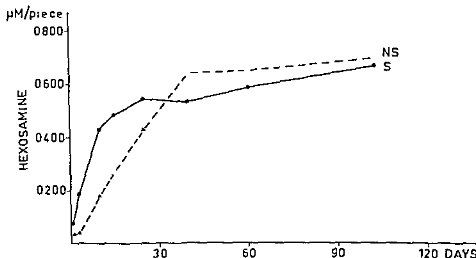


Fig 37 Amounts of hexosamines in saline soluble (S) and saline insoluble (NS) mucopolysaccharides in granulomas of different ages

The proportions of chondroitin sulphates in saline soluble and saline insoluble MPS are presented in Fig 38 A lower content of insoluble chondroitin sulphate and a higher content of soluble chondroitin sulphate were detected 3 days after the implantation of the cellulose sponges The

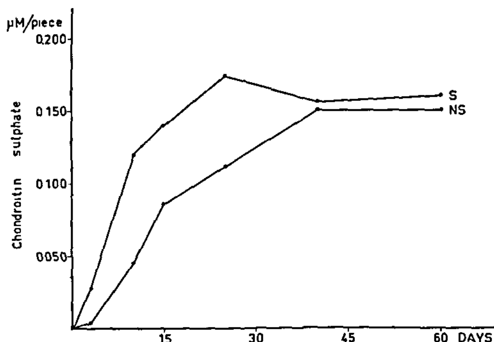


Fig 38 Amounts of saline soluble (S) and saline insoluble (NS) chondroitin sulphates in granulomas of different ages The amounts of saline-soluble chondroitin sulphates are mean values from Figures 31 and 32 and those of saline insoluble chondroitin sulphates mean values from Figures 35 and 36

content of the saline soluble chondroitin sulphate in the granulomas increased more rapidly than that of the saline insoluble chondroitin sulphate but both contents were high in old granulomas After the 40th day the content of chondroitin sulphate was almost the same in both fractions

Fig 39 presents the amounts of hexosamines in soluble and insoluble fractions containing neutral sugars The saline insoluble fraction increased with age and it can be supposed that this increase was due to an increase in the content of polysaccharide resembling keratosulphate because the amounts of hexosamine and galactose increased with age The amount of the soluble fraction was high already during the first few days this was perhaps due in a high degree to the presence of glycoproteins derived from tissue fluid but because the content of hexosamine in this fraction also increased with age the increase can be thought to arise from the polysaccharide resembling keratosulphate Because the content of neutral sugars was highest in the first few days the content of glycoproteins or neutral mucopolysaccharides must decrease with age

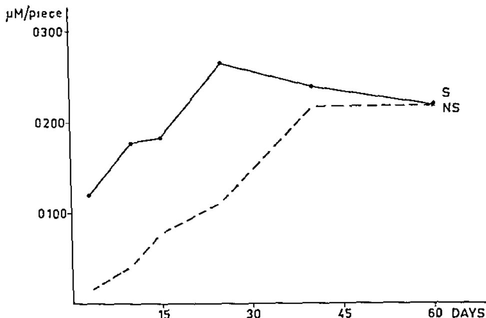


Fig 39 Amounts of hexosamines in fractions containing kerato ulphate like poly saccharide and glycoproteins or neutral mucopolysaccharides The amounts are the means of the results obtained after fractionation of cetylpyridinium complexes of the muco polysaccharides on a cellulose column and column chromatography of the mucopoly saccharide fractions on DEAE Sephadex (Figs 31 3? 35 and 36)

DISCUSSION

DISCUSSION OF METHODS

The complete separation of different mucopolysaccharides by column chromatographic methods is difficult. Electrophoresis on cellulose acetate offers an additional method for the separation of mucopolysaccharides. It is however very difficult to make the electrophoretic method quantitative. The electrophoretic method is rapid and useful for the analysis of MPS when these are available in amounts of about 5—10 micrograms, whereas relatively large amounts are required when MPS are fractionated by elution from columns of cellulose or DEAE Sephadex. Disadvantages of the electrophoresis are that the mobilities of keratosulphate and heparitin sulphate are equal at pH 8.6 and that chondroitin sulphates A, B and C cannot be separated from each other. However, it is possible to analyse the monosaccharide components of the fractions by gas chromatography which separates keratosulphate and heparitin sulphate and the iduronic acid of chondroitin sulphate B in the chondroitin sulphate fraction.

It is not possible to analyse quantitatively all sugars in a mixture by colorimetric methods. The gas chromatographic method for the quantitative determination of glucosamine and galactosamine is simple and reliable. The determination of neutral sugars by gas chromatography is more difficult because hexoses are easily destroyed by acid hydrolysis. Difficulties are also encountered in the analysis of sugar mixtures due to the great number of peaks arising from the different isomers of the monosaccharides. In the present study the total amount of neutral sugars was determined by a colorimetric method but the individual neutral sugars were determined by gas chromatography. The determination of uronic acid by GLC is difficult because it is not easy to isolate it from the polysaccharides without decarboxylation. It was however possible to use GLC to detect uronic acids in polysaccharide hydrolyzates.

COMPARISON OF PRESENT AND PREVIOUSLY REPORTED RESULTS

Experimental granulomas

The early synthesis of MPS in granulomas has been established by many investigators (Slack 1957, Chvapil and Cmuchalova 1961 Trnavsky *et al* 1961) The results of this study are in agreement with this conclusion The amount of soluble MPS increases rapidly after the first day following the implantation of the sponge and the amount of insoluble MPS after the third day (Fig 37) The formation of collagen fibres occurs later than the formation of MPS (Kao *et al* 1957 Woessner and Boucel 1961) Insoluble collagen in the granulomas increases most rapidly in the 2nd and 3rd weeks after the implantation of the cellulose sponges (Viljanto 1964) Robertson and Hinds (1956) Noble and Boucel (1958) and Daniel Moussard and Quesson (1961) found that the saline soluble hexosamine content of granulomas increased at first and decreased later while the saline insoluble hexosamine content increased with age The results of this study do not confirm the decrease of the saline soluble fraction with age This difference may be due to the purification of the saline soluble extract by a proteolytic enzyme and to the removal of protein carried out in this study The increase in the saline insoluble MPS content with age is in agreement with the earlier results Noble and Boucel (1958) noted that galactosamine appeared first in the insoluble and then in the soluble MPS fraction In this study galactosamine was found to be present in the soluble MPS fraction on the first day while a very small amount of galactosamine was found in the insoluble MPS fraction on the first three days Because galactosamine is one of the components of glycoproteins its presence in the soluble fraction is easily understood Hyaluronic acid has been stated to be the predominating MPS in young granulomas because the hexosamines consist mainly of glucosamine (Noble and Boucek 1958, White *et al* 1961) Also in the present study glucosamine was found to predominate in young granulomas but the analysed amount of hyaluronic acid after the fractionation of the MPS was surprisingly small the greatest amount of glucosamine being found in the fraction containing possibly keratosulphate and glycoproteins or neutral muco polysaccharides Viljanto (1964) found the content of hexosamine to be maximal on the 5th day and that of uronic acid on the 7th day His results were based on the determination of total hexosamines and uronic acids in the tissue without prior extraction and purification of the MPS Bollet *et al*

(1958) found that the ratio of uronic acid contents determined by the carbazole and orcinol methods decreased with age and suggested that the proportion of chondroitin sulphate B is higher in older granulomas. In the present study chondroitin sulphate B was not detected either by gas chromatography or by optical measurements. However, the results of the present study show that the ratio of uronic acid contents determined by the carbazole and orcinol methods is low in the chondroitin sulphate fractions, especially in fractions from older granulomas. When uronic acid is determined by the carbazole method, the molar ratio of uronic acid to hexosamine is close to unity whereas the orcinol method gives too high a ratio. The high content of uronic acid determined by the orcinol method may be caused by a nonspecific reaction. Berenson and Dalferes (1960) identified hyaluronic acid, heparitin sulphate and chondroitin sulphates A and B in granulomas produced by injecting turpentine oil into tissues. The identification of chondroitin sulphate B was based on the measurement of optical rotation and iduronic acid was identified by paper chromatography. Heparitin sulphate was not found in the present study. This may be due to the differences in the composition of MPS in granulomas produced by different methods. Delaunay and Bazin (1964) found that, in agreement with the results of this study, the amount of glycoproteins increased more rapidly than the amount of MPS in granulomas.

Changes in mucopolysaccharide composition in granuloma and other tissues with age

Many investigators have attempted to distinguish between the carbohydrates which are present as constituents of the interfibrillary matter of connective tissue and those which are in some way combined in the fibres. Especially hyaluronic acid is easily extracted from tissues by water or salt solutions (Meyer 1954, Mathieson and Pierce 1963), whereas chondroitin sulphate is more firmly bound to the tissue residue (Houck and Jacob 1960). The fraction extracted with 0.15 N sodium chloride contains proteins, glycoproteins and MPS (Sweeny *et al.* 1963). Gersh and Catchpole (1949) reported that the ground substance becomes more difficult to extract as it develops. According to the results of the present study all the MPS fractions are to some extent extractable with saline. The extractable MPS form the principal part of the total MPS in young granulomas but their proportion decreases later. The large amount of neutral sugars in the saline soluble fraction in the first few days after implantation is probable derived from tissue fluid.

White *et al* (1961) found glycoproteins and MPS and Jackson *et al* (1960) a large amount of mucoproteins in granulating wounds The amounts of MPS in granulating wounds of different ages have not been determined Metachromatic staining reveals that the amount of MPS is a maximum in wound tissue on the fifth or sixth day (Dunphy and Udupa 1955), the amount of MPS is a maximum later in granulomas

Hyaluronic acid, keratosulphate and chondroitin sulphate have been identified in cartilage (Hall *et al* 1957) Keratosulphate has been found to increase and chondroitin sulphate to decrease in ageing cartilage, at the same time chondroitin sulphate A is replaced by chondroitin sulphate C (Kaplan and Meyer 1959, Buddecke and Sziegoleit 1964) The composition and changes in granulation tissue are very similar although chondroitin sulphate A was the principal if not the only chondroitin sulphate and a decrease of chondroitin sulphate with age was not found

Bertelsen (1962) found that the amount of MPS increased or remained constant in the aorta with increasing age but Clausen (1962) reported that the hexosamine content of the aorta decreased Hyaluronic acid and chondroitin sulphate C have been found to decrease with age while chondroitin sulphate B and heparitin sulphate increased (Kaplan and Meyer 1960) The composition of MPS in the aorta and the changes with age differ from those of MPS in granulomas Houck *et al* (1961) found that saline insoluble hexosamine appeared in the skins of rats weighing over 206 g This is surprisingly late compared with the early appearance of insoluble hexosamines in experimental granulomas in rats Hyaluronic acid and chondroitin sulphate C are both present in large amounts in embryonic skin while adult skin contains more chondroitin sulphate B than hyaluronic acid (Loewi and Meyer 1958)

In conclusion it may be said that the amounts of keratosulphate and chondroitin sulphate increase and the relative and possibly the absolute amounts of hyaluronic acid decrease with age in tissues The changes in the composition of the MPS in granulomas are well correlated with these changes and hence experimental granulomas offer possibilities for the investigation of the ageing process in tissues

THE RATIOS OF INSOLUBLE AND SOLUBLE COMPONENTS

As can be seen in Figs 37—39 the amounts of total soluble MPS, chondroitin sulphate and the fraction containing polysaccharide resembling keratosulphate and glycoproteins or neutral mucopolysaccharides increased more rapidly than the amounts of the insoluble components The

(1958) found that the ratio of uronic acid contents determined by the carbazole and orcinol methods decreased with age and suggested that the proportion of chondroitin sulphate B is higher in older granulomas In the present study chondroitin sulphate B was not detected either by gas chromatography or by optical measurements However, the results of the present study show that the ratio of uronic acid contents determined by the carbazole and orcinol methods is low in the chondroitin sulphate fractions especially in fractions from older granulomas When uronic acid is determined by the carbazole method, the molar ratio of uronic acid to hexosamine is close to unity, whereas the orcinol method gives too high a ratio The high content of uronic acid determined by the orcinol method may be caused by a nonspecific reaction Berenson and Dalferes (1960) identified hyaluronic acid heparitin sulphate and chondroitin sulphates A and B in granulomas produced by injecting turpentine oil into tissues The identification of chondroitin sulphate B was based on the measurement of optical rotation and iduronic acid was identified by paper chromatography Heparitin sulphate was not found in the present study This may be due to the differences in the composition of MPS in granulomas produced by different methods Delaunay and Bazin (1964) found that in agreement with the results of this study the amount of glycoproteins increased more rapidly than the amount of AMPS in granulomas

Changes in mucopolysaccharide composition in granuloma and other tissues with age

Many investigators have attempted to distinguish between the carbohydrates which are present as constituents of the interfibrillary matter of connective tissue and those which are in some way combined in the fibres Especially hyaluronic acid is easily extracted from tissues by water or salt solutions (Meyer 1954 Mathieson and Pierce 1963), whereas chondroitin sulphate is more firmly bound to the tissue residue (Houck and Jacob 1960) The fraction extracted with 0 15 N sodium chloride contains proteins glycoproteins and MPS (Sweeny *et al* 1963) Gersh and Catchpole (1949) reported that the ground substance becomes more difficult to extract as it develops According to the results of the present study all the MPS fractions are to some extent extractable with saline The extractable MPS form the principal part of the total MPS in young granulomas but their proportion decreases later The large amount of neutral sugars in the saline soluble fraction in the first few days after implantation is probable derived from tissue fluid

SUMMARY

The ageing of experimental granulation tissue was studied by implanting viscose cellulose sponges under the dorsal skins of rats and analysing chemically the mucopolysaccharides in granulomas of different ages. Implants, 40—80 in number, were removed after 1, 3, 10, 15, 25, 40, 60 and 102 days. The composition of the saline soluble mucopolysaccharides and the composition of the saline insoluble mucopolysaccharides and their changes with age were determined separately. The mucopolysaccharides were liberated from their protein complexes with papain, the protein was precipitated with trichloroacetic acid and the mucopolysaccharides were precipitated with ethanol. The mucopolysaccharides were analysed chemically by determining their contents of hexosamines, uronic acids, neutral sugars, sulphate, sialic acid and nitrogen by colorimetric methods and then monosaccharide compositions by gas chromatography. The mucopolysaccharides were fractionated (1) as their cetylpyridinium complexes on a cellulose column, (2) by chromatography on a DEAE Sephadex anion exchanger and (3) by electrophoresis on cellulose acetate. The eluted fractions were analysed chemically.

The main results may be summarized as follows.

1. The results relating to saline soluble mucopolysaccharides (pp. 51—55). An early rise in the amounts of neutral sugars and also of hexosamine was observed. Also sialic acid was detected on the first and third day. On the third day after the implantation of the sponges acid mucopolysaccharides, both hyaluronic acid and chondroitin sulphates were present. The content of chondroitin sulphates subsequently increased rapidly until the 25th day, after which it changed very little (Figs. 31—32). The content of hyaluronic acid increased likewise with age but remained relatively low (Figs. 31—32). The fraction containing a polysaccharide resembling keratosulphate and polysaccharides from glycoproteins or neutral mucopolysaccharides predominated among the soluble mucopolysaccharides (Figs. 31—32), but the components of this fraction could not be determined separately. The presence of relatively large amounts of glucose and mannose in addition to galactose (Fig. 30), however, shows that this fraction consists mainly of glycoproteins or neutral mucopolysaccharides, at least during the first few days, although the polysaccharide resembling

keratosulphate could be identified in the fraction by electrophoresis The glucosamine content increased more rapidly than that of galactosamine in young granulomas (Fig 30)

2 The results relating to saline insoluble mucopolysaccharides (pp 55—59) Small amounts of amino sugars and neutral sugars were found on the first day after the implantation of the sponges and chondroitin sulphate was detected already on the third day (Figs 35—36) The content of chondroitin sulphates increased until the 40th day and remained approximately constant later Insoluble hyaluronic acid was also present in low proportion, but no increase with age was observed The fraction containing neutral sugars was small on the first few days and then increased steadily Galactose was the predominating neutral sugar especially in older granulomas and thus the increase in this fraction can be ascribed to the formation of polysaccharide resembling keratosulphate

3 The amounts of total soluble mucopolysaccharides soluble chondroitin sulphate and the soluble fraction containing polysaccharide resembling keratosulphate and glycoproteins or neutral mucopolysaccharides increased more rapidly than the corresponding insoluble components (Figs 37—39) The insoluble mucopolysaccharides amounted to about 10—20 % of the soluble mucopolysaccharides on the third day after the implantation of the sponges and then increased steadily until the 40th day, when the amounts of both fractions were approximately equal

4 For the characterization of chondroitin sulphates N acetylhexosamines liberated from them by hyaluronidase were determined (Table II), the optical rotations of the chondroitin sulphate samples (Table III) were measured and the uronic acids of the chondroitin sulphates were analysed by gas chromatography (Fig 26) The determinations showed that chondroitin sulphate A predominated The amount of chondroitin sulphate C was very small if it was present at all, and chondroitin sulphate B was not detected

The results are discussed and compared with earlier results for experimental granulomas The changes in the compositions of mucopolysaccharides in granuloma and other tissues with age are discussed

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 309

THE MOLECULAR STRUCTURES OF VERTEBRATE SKIN COLLAGENS

A COMPARATIVE STUDY

BY

JARMO PIKKARAINEN

TURKU 1968

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SUPPLEMENTUM 300

DEPARTMENT OF MEDICAL CHEMISTRY UNIVERSITY OF TURKU FINLAND

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To the Memory of
My Father

Turku Kirjapaino Polytypos 1968

CONTENTS

SYMBOLS AND ABBREVIATIONS

I	*ionic strength*
T_D	denaturation temperature of solubilized collagen
T_s	shrinkage temperature
Δ	difference
$[\alpha]_\lambda$	specific rotation at wavelength λ
λ λ	constants in Drude and Moffitt Yang equations
TC	tropocollagen
NSC	*neutral salt soluble collagen*
AC	acid soluble or acid citrate buffer soluble collagen
IC	insoluble collagen
SLS	segment long spacing form of reconstituted collagen
CM cellulose	carboxymethylcellulose

INTRODUCTION

Mutation and natural selection are the two fundamental processes in evolution which occur at organ as well as molecular levels in living organisms The genetic information which determines all the properties of an organism can be found in the nucleotide base sequence of its DNA which is reflected in the amino acid sequences of proteins

Collagen is the principal protein of connective tissue Fibroblasts synthesize the monomeric collagen unit tropocollagen and secrete it into the extracellular space where the tropocollagen molecules associate to form collagen fibrils according to a certain pattern

The formation of connective tissue has been necessary for the development of specialized tissues and organs Collagen is found in all animal phyla except arthropods and certain classes of coelenterata The amount of collagen varies greatly from one species to another In mammals collagen is the most abundant protein as it comprises about one third of the body protein Certain invertebrates such as horny sponges and the sea cucumber contain even higher proportions of collagen

The purpose of the present study was to obtain information about the phylogeny of collagen and to define the essential features of collagen structure Collagen was prepared from 12 vertebrate species chosen to represent the phylogenetic tree These collagens were investigated by a variety of methods to obtain knowledge of the primary, secondary, tertiary and quaternary levels of collagen structure In the interpretation of data in terms of phylogeny care must be exercised and the following words of Florkin (1965) should be remembered 'In the present situation we must adopt the methodical rule to be led by the knowledge of phylogeny in our search for biochemical evolution rather than to be led by biochemistry into the discovery of new aspects of phylogeny The Ariadne s thread of comparative biochemistry *can only be the knowledge of phylogeny* in which is integrated the treasure of knowledge accumulated by generations of naturalists familiarized with living organisms

LITERATURE REVIEW

OUTLINES OF MOLECULAR EVOLUTION

A change in a gene leading to mutation can manifest itself in several ways in DNA. Single base replacement alters the corresponding amino acid in the polypeptide chain except when the codon is degenerate. Terminal additions and deletions of amino acids, *e.g.* in cytochrome *c* chains have been described (Margoliash and Smith 1965). During the evolutionary development of haemoglobin deletions are believed to have occurred because sequence gaps have been found in haemoglobin chains (Dixon 1966). Ingram (1961) put forward the hypothesis that the formation of new polypeptide chains in globin was a result of a complete gene duplication which duplication may also be partial as in haptoglobin Hp2α (Dixon 1966).

As a consequence of mutation the function which is a property of the three dimensional structure may remain unchanged or change over a very wide range, and even cease entirely (Margoliash 1963).

Many methods have been used in the analysis of proteins for phylogenetic purposes. The determination of the complete amino acid sequence would yield the most comprehensive information but at the moment the primary structures of only 10—15 proteins are known. Chromatography or 'finger printing' of peptide fragments was used by Ingram (1958) to locate differences in various haemoglobin. Immunochemical analyses were performed by Manski, Halbert and Auerbach (1964) in their studies of the phylogeny of lens protein.

When the results of protein analyses are discussed in relation to taxonomic aspects one must take into account the fact that only one gene has been investigated. Difficulties may also arise from the lack of homology of proteins (Florkin 1966) or from the dependence of functional similarity on convergent evolution (Mayr 1964, Zuckerkandl and Pauling 1965). Some erroneous conclusions may also be related to the translation of DNA to the amino acid sequence and *vice versa* that are due to degeneracy in the genetic code and to the non expressed areas in the DNA sequence (Dixon 1966).

STRUCTURE OF COLLAGEN

A number of review articles (Grassmann 1960, Harkness 1961, Harrington and von Hippel 1961, Gross 1963, Harding 1964, Grassmann *et al.* 1965, Harding 1965, Zonta and Campani 1965), books (Gustavson 1956, Veis 1964, Reich 1966), review monographs edited by Hall (1963, 1964, 1965) and symposia (Stainsby 1958, Page 1959, [illegible] 1961, Jackson *et al.* 1965, Comte 1966) contain in summarized form the information on collagen and connective tissue published in the last ten years.

The tropocollagen macromolecule is the basic structural unit of collagen In the native form it is a rigid rod shaped particle 2800 Å long and 14 Å in diameter and has a molecular weight of about 300 000 as revealed by physico-chemical and electron microscopic studies of soluble collagens

The molecule is composed of three polypeptide chains each forming a left handed helix The chains are further coiled into a three stranded ropelike superhelix (triple helix) These configurations are maintained mainly by hydrogen bonds Along the axis of the tropocollagen molecule there are sections of crystalline structure composed of nonpolar amino acids These sections are separated by amorphous structures composed mostly of polar amino acid

The arrangement of the chains in the tropocollagen molecule enables the formation of two kinds of collagen structure (Rich and Crick 1961) The triple helical structure presupposes that *in each chain glycine is in the position nearest the axis and the* pyrrolidine rings point outwards from the molecul In collagen I the hydroxyl group of hydroxyproline is directed towards the axis whereas it points away from the axis in collagen II

Amino acid composition

Very few analyses of amino acid compositions of collagens of different vertebrates *were performed in the first half of this century Usually one or two amino acids were* determined in different gelatin The samples were often impure and were pretreated with enzymes or alkalies that are known to modify collagen Eastoe and Leach (1958) presented amino acid compositions of 16 different species in which they express the results in residues per 1000 residues The effect of non protein impurities was thus eliminated and a comparison of various proteins was possible Reviews on amino acid compositions of collagens have been presented later by Piez and Likins (1960) Gross (1963) and Borasky (1963)

Table I presents previously reported data on amino acids of collagens of vertebrate skins Only data obtained by ion exchange chromatography are included They reveal *e g* that the amount of hydroxyproline and proline increases whereas that of methionine decreases in the course of evolution The hydroxyl group content has remained constant owing to a decrease in the number of serine and threonine residues as established already by Beveridge and Lucas (1944) by rather inaccurate colour reactions

In order to find out what is essential to collagen structure, the variation in amino acid composition was calculated (Table II) In accordance with presented models of collagen glycine accounts *for one third of the residues The contents of* acidic basic and hydroxy amino acids are separately constant despite the great variation in the proportions of individual amino acids within these groups (see Discussion)

Table I

Amino acid compositions of various vertebrate collagens and gelatins

The values are numbers of amino acid residues per 1000 residues as determined by ion exchange chromatography

	Man	Cow	Pig	Whale	Rat	Rabbit	Wallaby
Hydroxyprolines (3 & 4)	93	90	93	89	93	105	93
Aspartic acid	46	46	46	46	45	50	49
Threonine	18	17	18	24	20	23	20
Serine	36	35	35	41	43	39	39
Glutamic acid	75	6	72	70	71	71	73
Proline	127	127	131	128	121	127	119
Glycine	327	330	328	326	331	311	320
Alanine	110	112	111	110	106	105	113
Half cystine (and cysteic acid)	<0.5	<0.5	<0.5	<0.5	—	<0.5	<0.5
Valine	25	21	24	21	24	24	23
Methionine (and sulphoxides)	6	5	5	5	8	7	7
Isoleucine	10	12	10	11	10	13	9
Leucine	25	25	24	25	24	25	26
Tyrosine	4	4	3	3	3	3	4
Phenylalanine	13	13	14	13	12	14	16
Hydroxylysines	8	6	7	6	7	5	8
Ornithine	—	<0.5	—	—	—	—	—
Lysine	25	26	26	26	27	29	25
Histidine	5	5	5	6	5	6	5
Arginine	50	50	48	50	50	47	51
Acidic amino acids	118	122	118	116	116	121	122
Basic amino acids	88	87	86	88	89	87	89
Amide N	41	43	38	26	41	40	44
Imino acids	220	217	224	217	214	229	212
Hydroxy amino acids	159	152	156	163	166	171	164
Nonpolar amino acids	643	645	647	639	636	626	631

For the occurrence of ornithine among the amino acids of collagen the reader is referred to the articles of Hamilton and Anderson (1954), Hirs, Stein and Moore (1954) and Murray *et al.* (1965).

The numbers are average values for several specimens of collagen as follows: two *human beings* (Bornstein and Piez 1964, Eastoe 1955); eleven *cows* (Cooper and Davidson 1965, Eastoe 1955, Grassmann, Hannig and Schleyer 1960, Grassmann, Nordwig and

	Python	Crocodile	Toad	Lungfish	Cod	Carp	Pike	Sturgeon	Shark
9	102	93	78	75	55	78	70	82	66
8	48	46	55	46	51	4,	54	48	43
9	18	22	26	25	23	-,	-5	29	24
9	43	4?	66	43	69	40	41	50	55
4	62	73	78	,7	74	71	81	71	68
9	119	128	110	127	97	118	129	102	106
1	316	3?4	301	319	343	3?5	328	337	337
5	125	114	98	127	110	1?2	114	119	112
—	<0 5	<0 5	<0 5	<0 5	—	—	—	<0 5	<0 5
0	20	15	?2	?0	19	18	18	18	25
6	6	6	9	4	18	14	12	9	15
11	1-	11	14	11	10	11	9	11	17
24	26	20	29	23	21	22	-0	18	25
3	2	3	6	1	4	3	2	-	2
14	14	18	19	15	11	14	14	14	13
10	4	5	4	6	7	7	8	11	6
—	—	—	—	—	—	—	—	—	—
19	?8	-5	29	?4	2,	26	2?	22	?5
4	5	5	7	5	9	5	8	5	10
45	50	50	49	5-	5?	??	45	52	51
2-	110	119	133	123	125	118	135	119	111
78	87	85	89	87	95	89	83	90	92
44	2?	2?	5?	45	46	34	42	41	34
-8	221	221	188	02	152	196	199	184	172
60	1?9	165	180	150	158	154	146	174	153
650	638	636	602	646	6?9	645	644	6-8	650

Veis and Schlucter 1964 Veis and Anesey 1965) two *pigs* (Eastoe 1955 Eastoe 1961) one *whale* (Eastoe 1955) two *rats* (Schlueter and Veis 1964), four *rabbits* (Jackson Leach and Jacobs 1958) one *wallaby* (Eastoe 1955) one *chicken* (Leach 1957) one *python* (Leach 1957) one *crocodile* (Leach 1957) one *toad* (Leach 1957) two *lung fishes* (Eastoe 1957) two *cods* (Piez 1964 Piez 1965) two *carps* (Piez and Gross 1960 Piez Eigner and Lewis 1963) one *pike* (Piez and Gross 1960) one *sturgeon* (Eastoe 195) and three *sharks* (Eastoe 195, Lewis and Piez 1964 Piez *et al* 1963)

Table II

Variation in the amino acid contents of various vertebrate collagens and gelatins

The values are based on the data in Table I

	Range	Δ	Mean	Δ/Mean
Hydroxyprolines (3 & 4)	55—102	47	78.5	0.60
Aspartic acid	43—55	12	49	0.24
Threonine	17—29	12	23	0.52
Serine	29—69	40	49	0.82
Glutamic acid	62—81	19	71.5	0.[illegible]
Proline	97—131	34	114	0.30
Glycine	301—343	4	32	0.13
Alanine	98—127	29	112.5	0.26
Half cystine (and cysteic acid)	<0.5	—	—	—
Valine	15—25	10	20	0.50
Methionine (and sulphoxide)	4—18	14	11	1.27
Isoleucine	9—17	8	13	0.62
Leucine	18—29	11	23.5	0.47
Tyrosine	1—6	5	3.5	1.43
Phenylalanine	11—19	8	15	0.53
Hydroxylysines	4—11		7.5	0.92
Ornithine	<0.5	—	—	—
Lysine	19—29	10	24	0.42
Histidine	4—10	6	7	0.86
Arginine	45—52		48.5	0.14
Acidic amino acids	*110—135*	25	122.5	0.20
Basic amino acids	78—95	17	86.5	0.20
Amide N	22—52	30	37	0.81
Imino acids	155—229	74	190.5	0.40
Hydroxy amino acids	146—180	34	163	0.21
Nonpolar amino acid	602—650	48	626	0.08

Optical properties

The total optical rotatory power of a native protein molecule depends on the amino acid composition and on the helix content of the protein. Collagen has a unique amino acid composition involving high contents of glycine and especially imino acids which makes the triple helical structure possible (Szent-Györgyi and Cohen 195[illegible]). This structure in turn is responsible for the exceptionally great levorotation of native collagen. The rotatory properties are determined by the left-handed helical charac

Table III

Optical properties of vertebrate tropocollagen solutions

	Solvent	$-[\alpha]^{H}_{589}$	$-[\alpha]^{D}_{589}$	$-[\alpha]^{H}$	$-[\alpha]^{D}$	λ^{H}	λ^{D}	References
Mammals								
Man	0 15M acetate pH 4 8	428	142	2540	831			1
	0 15M citrate, pH 3 7	415						2
Cow	0 15M citrate pH 3 7	415	135					3
		400						4
				1000	360			5—6
	"			1305	468 *			7
	"			1330	460			8
	0 1M citrate pH 3 5	440	140					9
	0 15M acetic acid	415						10
	0 05 % acetic acid			1330 *	460	195		11—12
Rat	water	289	118			217	17	13
	citrate pH 3 7 I 0 075	409	135					14
		416	133					15
		340	131					16
Birds								
Chick	0 5 % acetic acid	400						17
Bony fishes								
Perch	citrate pH 3 7 I 0 075	400	1 8					14
		39	120					15
Cod		397	116					14
		380	119					15
	0 15M citrate pH 3 0		118					18
	0 1M citrate pH 3 6	349	107					19
	0 1M lactate pH 3 6	403						19
	0 1M tartrate pH 3 6	389						19
	0 1M citrate pH 3 4	373	113					20
Eel	citrate pH 3 7 I 0 075	398	1 6					14
Carp	0 15M citrate pH 3 7					205±15		21
	citrate pH 3 7 I 0 075	530				204		13
	0 1M acetic acid							
	0 1M KCl pH 2 8			1350	460			22
Herring	citrate pH 3 7 I 0 075	408	125					14
Cartilaginous fishes								
Dogfish	0 15M citrate pH 3 5	345	12					23
		398	24			213		24

313 nm 365 or 367 nm 405 nm

1 Bornstein and Piez (1964) *2* Bakerman (1961a) *3* Doty and Nishihara (1958) *4* Piez *et al* (1964) *5* Kuhn (1963) *6* Kuhn Fietzek and Kuhn (1966) *7* Rubin *et al* (1965) *8* Drake *et al* (1966) *9* Steven and Tristram (1962) *10* Cooper and Davidson (1965) *11* Altgelt Hodge and Schmitt (1961) *12* Fujimori (1966) *13* Harrington (1958) *14* Burge and Hynes (1959a) *15* Burge and Hynes (1959b) *16* Flory and Weaver (1960) *17* Levene and Gross (1959) *18* Astrup Marko and Young (1958) *19* Young and Lorimer (1960) *20* Young and Lorimer (1961) *21* Cohen (1955) *22* Veis and Drake (1963) *23* Lewis and Piez (1961) *24* Lewis and Piez (1964)

teristics of the individual strands rather than by the right handed superhelix formed by the three polypeptide strands (Urnes and Doty 1961) When the organized structure of native collagen is de troyed, e g by heating the negative rotation diminishes to a value characteristic of its amino acid compo ition

Measurements of optical rotation at different wavelengths the optical rotatory di per ion have revealed that the disper ion by both native and denatured collagen is of a simple type and can be represented by a one term Drude equation over the region 400—700 nm (Harrington *et al* 1961 von Hippel and Wong 1963 b)

Table III pre ents values of specific rotation and optical dispersion for solutions of collagens from different species The origin of the collagen does not seem to affect the optical properties

Components

Denaturation of tropocollagen solutions by heat or competitors for hydrogen bonds (urea, rhodanide) destroys the organized structure Mathews, Kulonen and Dorfman (1954) demonstrated first the separation of collagen into two components on heating its solution and a cribed the results to a reversible dissociation of procollagen mono mer The e two components have been later named α and β The β component has twice the molecular weight of the α component and represents a combination of two α component Ultracentrifugation of acid soluble collagens yielded a omponent named γ which sedimented more rapidly than the β component (Gra smann Hannig and Engel 1961 Altgelt *et al* 1961) Veis Ane ey and Cohen (1960) extracted a till heavier component which they named δ from bull hide corium with hot water Molecular weight determinations revealed that the γ-component is tropocollagen that is a com bination of three α chains while the δ component is composed of four cross linked γ components (Vei Anesey and Cohen 1962)

Piez *et al* (1961) first demonstrated that purified tropocollagen from rat skin contains two types of α chains named $\alpha 1$ and $\alpha 2$ and that it has the composition $\alpha 1 \alpha 1$-α The exi tence of a third chain type in cod skin collagen was later proved by Piez (1964 1965) and he named the new chain $\alpha 3$ The existence of two different $\alpha 1$ chains in guinea pig collagen was propo ed by Kulonen *et al* (1965) The noniden tity of the three α chains in rat skin and chick bone collagens has al o been reported (Heidrich and Wynston 1965 Francois and Glimcher 1966)

A uming three different α chains there should exist three types of β components of intramol cular origin namely β_{2} β and β_{n} Up to the present only two such β-components (named β and β_{2}) have been identified probably because of the very clo e chemical similarity of the $\alpha 1$ and $\alpha 3$ chains Extraction of mammalian skin with 5 M guanidine olution has yielded a new type of component, β_{n} which is believed to be of intermolecular origin (Born tein Martin and Piez 1964)

A succe ful separation of collagen components of equal mole ular weight can at pre ent be effe ted only by chromatography on carboxymethylcellulose columns (Kess ler Rosen and Leven on 1960 Piez *et al* 1961, Schleyer 1962) or by gel electrophoresis (Nantö Maat la and Kulonen 1963 Peich 1964 Nantö Pikkarainen and Kulonen 1965)

Collagen structure in various vertebrates

Histology Microscopical studies have revealed that the collagen fibres in the skins of aquatic animals are arranged sid by si le in laminae whereas land living

vertebrates have a much more randomly organized pattern of collagen bundles (Gross 1963)

Molecular dimensions Investigations of molecular weights and molecular dimensions of native tropocollagen with the aid of sedimentation analysis light scattering measurements viscometry and electron microscopy have not revealed any differences in the tropocollagens of calf (Doty *et al* 1958 Engel and Beier 1963) cod (Young and Lorimer 1961), carp (Hall and Doty 1958) and spiny dogfish (Lewis *et al* 1964)

The molecular weights of the α β and γ-components do not differ from each other in man (Bornstein and Piez 1964) calf (Doty *et al* 1958 Piez Weiss and Lewis 1960 Grassmann *et al* 1961a Engel *et al* 1963), rat (Orekhovitch and Shpikiter 1958a), cod (Piez 1965) carp (Boedtker and Doty 1956) or spiny dogfish (Lewis *et al* 1964)

Components Determinations of the molar ratios of the α and β components in soluble collagens have shown that there are mainly α chains in collagens soluble in neutral salt solution whereas the ratios of α and β components in collagens soluble in acid buffer solution are equal This is in agreement with the recent view that the neutral salt soluble collagen represents a recently synthesized collagen and that the number of cross links in collagen increases with age as found, *e g* by starch gel electrophoresis (Heikkinen and Kulonen 1964) Bakerman (1964), however, was not able to demonstrate any differences in the ratio of α and β-components in soluble kin collagens from human beings of different age

Since the discovery of the dissimilarity of the α chains, CM cellulose chromatography or/and gel electrophoresis have been used to study the compositions of collagen chains in the following vertebrates man (Bornstein and Piez 1964), cow (Schleyer 1962 Heidrich *et al* 1965 Veis and Anesey 1965) rat (Piez *et al* 1961, Nänto *et al* 1963) lathyritic chick (Tanzer Monroe and Gross 1966) cod (Piez 1965) carp (Piez *et al* 1963) and spiny dogfish (Piez *et al* 1963 Lewis *et al* 1964)

The existence of α β and γ components has been confirmed in all these species In determinations of the approximate component distributions in acid soluble collagens of rat skin and carp ichthyocol Piez *et al* (1963) found that the ratio of $\alpha1$ to $\alpha2$ varies from 2 0 to 2 3 whereas the acid soluble collagen of young spiny dogfish skin contains about ten times more $\alpha1$ than $\alpha2$ The behaviour of dogfish collagen on CM cellulose column chromatography was also quite different and a high concentration of salt was required to elute the components from the column The molecular structure $(\alpha1)_2\alpha^2$ is generally accepted for tropocollagen regardless of species Cooper and Davidson (1965) however have proposed the structure $\alpha1(\alpha^2)$ for the collagen of premature calves whereas the structure of collagen in a full term calf is $(\alpha1)$ $\alpha2$

Preliminary reports on the dissimilarity of the component compositions of guinea pig pike rayfish hagfish and lamprey collagens have been published (Pikkarainen and Kulonen 1964 1965 1967 Pikkarainen Rantanen and Kulonen 1966)

Stability of collagen structure

Stability of the helix Investigations on the stability of the secondary structure of the native collagen molecule which is maintained mainly by hydrogen bonds usually deal with processes associated with the collagen gelatin transitions

The collagen gelatin transition is a melting process in which collagen changes into a disorganized random coil (Flory and Garrett 1958) This transition may be detected at various levels of structural organization, *e g* by measuring the thermal shrinkage

Table IV

Denaturation temperatures of vertebrate collagens in the solid (T_S) and dissolved (T_D) states

The T_S values are thermal shrinkage temperatures determined for skins in water or physiological saline. The T_D values were determined either by viscosimetry or polarimetry and refer to acid buffered tropocollagen solutions

	T_S	T_D	References
Mammals			
Man	60—6[illegible]	36—39	1—6
Ruminants	[illegible]9—[illegible]0	34—39	3 7—18
Pig	6[illegible]	—	19
Horse	62—64	—	9
Dugong	69	—	20
Badger	[illegible]9—60	—	9
Fin whale	6[illegible]—64	—	9
Rat	[illegible]9	37—38	3 [illegible]1—[illegible]
Rabbit	60—6[illegible]	—	9
Reptiles			
Crocodile	[illegible]9	—	19
Python	[illegible]7—[illegible]9	—	19
Lizard	60—6[illegible]	—	9
Amphibians			
Toad	54	—	19
Bony fishes			
Lungfish	63	—	[illegible]3
Flatfish	38—43	—	9
Perch	48—[illegible]	30—31	2—3 9 21 [illegible]4
Cod	37—4[illegible]	1[illegible]—1[illegible] (29)	3 7—9 11—12, 21 2[illegible]—2[illegible] (13)
Eel	[illegible]—[illegible]	[illegible]6	9 [illegible]1
Carp	49—[illegible]8	[illegible]9—3[illegible]	3 9 13 15 28
Pike	4[illegible]—[illegible]7	27	3 9 19 12
Herring	—	18	21
Sturgeon	50	—	[illegible]3
Cartilaginous fishes			
Shark	5[illegible]	29	[illegible]3 [illegible]
Dogfish	3[illegible]—41	16	3 29—31

In toluene [illegible] Hadler

1 Hall and Reed (19[illegible]) *2* Bigley (196[illegible]) *3* Rigby (1967), *4* Bakerman (1961a) *5* Bakerman (1961b) *6* Bornstein and Piez (19[illegible]4) *7* Gustavson (1942), *8* Gustavson

which is a macroscopic manifestation of collagen transformation or by the determination of the denaturation temperature of dissolved collagen

A comparison of the thermal stabilities of vertebrate collagens is presented in Table IV The values are relatively constant (shrinkage temperature in the range of 60 —70 and denaturation temperature 36 —39) except for fish collagens

Gustavson (1954b 1955) studied thermal shrinkage very carefully, especially that of skins of various fishes He suggested that the hydrothermal stability depends on the interchain hydrogen bonding of hydroxyl groups of hydroxyproline Burge and Hynes (1959b) proposed a fundamental relationship between the stability and the total amino acid content rather than the hydroxyproline content This was confirmed by Piez and Gross (1960) Maser and Rice (1962, 1963b) and Josse and Harrington (1964) According to Harrington (1964), the pyrrolidine residues stabilize the triple helical structure by decreasing the total configurational entropy change in the helix coil transition In addition to the stabilizing effect of hydrogen bonds between amide and carbonyl groups of adjacent strand water molecule chains may be of importance as they may form interchain hydrogen bonds (Berendsen and Migchelsen 1966) The effects of van der Waals forces as well as of cross links between the individual strands seem to be unimportant for stability (Harrington and McBride 1966)

Solubility of collagen The solubility of collagen can also be considered a measure of stability depending on the quaternary structure (cross linking) of the collagen. The tropocollagen molecules are in fibrous form in the tissue The fibres are bound by intermolecular cross links which are responsible for the relative insolubility of collagen in aqueous and organic solvents (Hormann 1962 Harding 1965) Collagen can however be separated into various artificial fractions by employing different solvents

The solubility of collagen in dilute acids has been known almost a century More attention was paid to soluble collagens after Russian workers showed that a part of skin collagen dissolves in an acid buffer solution The results of these studies on this procollagen' from several sources have been summarized by Orekhovitch and Shpikiter (1958b)

A portion of this procollagen was extracted from the tissues also by alkaline phosphate and neutral salt solutions The neutral salt soluble collagen is the precursor of mature collagen The biological significance of acid soluble procollagen is as yet unknown It has been considered not only a precursor of collagen but also a degradation product (In the present study the acid soluble or acid citrate buffer soluble collagen refers to collagen extracted from the skin after the removal of the neutral salt soluble collagen)

In comparisons of soluble and insoluble collagens very minute perhaps insignificant differences have been found *e g* in amino acid composition (Polatnick La Tessa and Katzin 1957 Jackson *et al* 1958 Orekhovitch *et al* 1960), molecular weight and molecular dimensions (Hannig and Engel 1961 Kawai *et al* 1966)

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The high solubility of fi h kin collagen on heating has been known for a long tim (Gustavson 1942) Table V pre ents data on the solubilities of skin collagens of ful grown vertebrates The show that the amount of native oluble collagen in mammalia skin is remarkably low compared to its amount in fi h skin

Table V
Contents and solubilities of native coll gens of skins of full grown vertebrates

	Percent collagen in		Percent collag n olubl in		References
	wet skin	dry skin	neutral salt olutions	acid buffer solutions	
Man	1 —16	40—95	0 11—0 56	0 0 —3 2	1—6
Cow	—	65—91	0 03	0 5—5	7—10
Guinea pig	—	33—68	7—8	1	13—15
Rat	15—26	40—75	0 05 —7 6	0 88 —6 7	16—27
Mou e	1—	43—58	0	2 3	27—29
Rabbit	15	37	15—4 3	5 33	15 30
Cod	—	74	—	62	31

Solubl colla en as a p rcentage of the dry weight of the kin
Soluble collagen as a percentage of the wet weight of th skin
1 Bakerman (1964) *2* I orting Holzmann and Kuhn (1964) *3* Harris an Sjoerd ma (1966) *4* Sobel *et al* (1958) *5* Clau en (1962) *6* Maj w ki Leja an Majewska (1964) *7* Grassmann (1960) *8* Veis An sey and Cohen (1960) *9* Crosl and Stain by (1962) *10* Smits *et al* (19) *11* Bowe Elliott and Mo s (1958) *12* V rzar (1960) *13* Gro s (1958) *14* Orekhovitch and Sh ter (1958) *15* Sakai (1960) *16* Wirtschafter and Bentl y (1962) *17* Hol (1964) *18* Kuh *et al* (1964a) *19* Kuhn *et al* (1964b) *20* Nimni (1964) *21* V (1964) *22* Mills and Bavetta (1966) *23* Kao H avel (1960) McGavack and Kao (1960) *25* Murray Watts and R Cadavid Dendu and Mancini (1963) *27* Dicker on and John (1964), Marchant (*29* Harkne s Harkness and James (1958) Nin Bavetta (19 *31* Young and Lorimer (1960)

The quantity of soluble collagen of th r (Gro s 1958b) and with the site from 1964 amount of acid soluble collag n depen d ti u that is extracted Ru s (1964) d to 95 % of bull hide collagen dissolv

In olubl collagen that remains be di olved as g latin Iikkarain n starch g l el ctrophoretic pattern of I reveal the existenc of α and β co molecular weight aggregat s have n hid corium in water at 60 (Veis *et a*

which is a macroscopic manifestation of collagen transformation or by the determination of *the denaturation temperature* of dissolved collagen

A comparison of the thermal stabilities of vertebrate collagens is presented in Table IV The values are relatively constant (hrinkage temperature in the range of 60 —70 and denaturation temperature 36 —39) except for fish collagens

Gustavson (1954b 1955) studied thermal shrinkage very carefully, especially that of kins of various fishes He suggested that the hydrothermal stability depends on the interchain hydrogen bonding of hydroxyl groups of hydroxyproline Burge and Hynes (1959b) proposed a fundamental relation hip between the stability and the total imino acid content rather than the hydroxyproline content This was confirmed by Piez and Cross (1960), Maser and Rice (1962, 1963b) and Josse and Harrington (1964) According to Harrington (1964) the pyrrolidine residues stabilize the triple helical stru ture by decreasing the total configurational entropy change in the helix coil transition. In addition to the stabilizing effect of hydrogen bonds between amide and carbonyl groups of adjacent strands water molecule chains may be of importance as they may form interchain hydrogen bonds (Berendsen and Migchel en 1966) The effects of van der Waals forces as well as of cross links between the indivi lual strands seem to be unimportant for stability (Harrington and McBride 1966)

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The high solubility of fish skin collagen on heating has been known for a long time (Gustavson 1949) Table V presents data on the solubilities of skin collagens of full grown vertebrates These show that the amount of native soluble collagen in mammalian skin is remarkably low compared to its amount in fish skin

Table V
Contents and solubilities of native collagens of skins of full grown vertebrates

	Percent collagen in		Percent collagen soluble in		References
	wet skin	dry skin	neutral salt solutions	acid buffer solutions	
Man	12—16	40—95	0 11—0 6	0 02 —3 2	1—6
Cow	—	65—91	0 03	0 5—5	7—10
Guinea pig	—	33—68	7—8	1	13—15
Rat	15—26	40—75	0 05 —7 6	0 88 —6 7	16—27
Mouse	1—7	43—58	0 2	2 3	27—29
Rabbit	15	37	2 15—4 3	5 33	15 30
Cod	—	74	—	62	31

Soluble collagen as a percentage of the dry weight of the skin
Soluble collagen as a percentage of the wet weight of the skin
1 Bakerman (1964) *2* Korting Holzmann and Kuhn (1964) *3* Harris and Sjoerdsma (1966) *4* Sobel *et al* (1958), *5* Clausen (1962), *6* Majewski Leja and Majewska (1964) *7* Grassmann (1960) *8* Veis Anesey and Cohen (1960) *9* Crosby and Stainsby (1962) *10* Smuts *et al* (1955), *11* Bowes Elliott and Moss (1958) *12* Verzar (1960) *13* Cross (1955) *14* Orekhovitch and Shpikiter (1958b), *15* Sakata (1960) *16* Wirtschafter and Bentley (1962) *17* Holzmann *et al* (1964), *18* Kuhn *et al* (1964a) *19* Kuhn *et al* (1964b) *20* Nimni and Bavetta (1964), *21* Verzar (1964) *22* Mills and Bavetta (1966) *23* Kao Hilker and McGavack (1960) *24* McGavack and Kao (1960) *25* Murray Watts and Ring (1961), *26* Cadavid Denduchis and Mancini (1963) *27* Dickerson and John (1964) *28* Hamer and Marchant (1957), *29* Harkness Harkness and James (1958) *30* Nimni DeGuia and Bavetta (1966), *31* Young and Lorimer (1960)

The quantity of soluble collagen varies with the nutritional state of the animal (Cross 1958b) and with the site from which the skin piece is taken (Verzar 1964) The amount of acid soluble collagen depends greatly on the particle size of the disintegrated tissue that is extracted Rigby (1964) and Kuntzel (1964) have demonstrated that up to 95 % of bull hide collagen dissolves after intensive homogenization

In soluble collagen that remains after thorough extraction of soluble collagens may be dissolved as gelatin Pikkarainen *et al* (1964) were able to demonstrate that the starch gel electrophoretic pattern of insoluble collagen of guinea pig skin heated to 40 reveals the existence of *α* and *β* components in addition to larger aggregates High molecular weight aggregates have also been found in extracts obtained on heating bull hide corium in water at 60 (Veis *et al* 1960)

THE PURPOSE OF THE PRESENT INVESTIGATION

The purpose of the present investigation was to obtain knowledge of the evolution of collagen Comparative chemical investigations of skin collagens of several vertebrates at different levels of protein structure were carried out to find answers to the following questions

1 Do the *amino acid compositions* of collagens correlate with animal evolution? What are essential features of collagen structure?

2 Has the *helical structure* remained constant during evolution as judged by the optical rotatory properties of tropocollagen solutions? How much does the *thermal stability* of the helical structure of collagens vary as measured by optical rotation and thermal contraction? How does the stability correlate with chemical structure?

3 Are the *components* of tropocollagen influenced by evolution? Is an ancestral polypeptide chain detectable?

4 Are the *solubilities* of skin collagens related to the evolutionary stage?

MATERIAL AND METHODS

SAMPLES OF SKINS

The specimens were collected alive when possible. Otherwise only fishes that had been dead at most one hour and had been stored meanwhile on ice were used. The samples included the following materials:

Cattle (*Bos taurus* L.), forehead and neck skin of a cow (age unknown) and a calf (age one week).

Guinea pig (*Cavia porcellus* (L.)), skins of 8 full grown (weighing 520—840 g, mean 650 g) and 15 growing (weight under 500 g) guinea pigs,

Chick (*Gallus domesticus* L., White Rock) skins of two broilers (weighing about 1.5 kg).

Ringed snake (*Natrix natrix* (L.)) skins of 5 snakes (lengths over 50 cm),

Toad (*Bufo bufo* (L.)) and *Frog* (*Rana temporaria* L.) skins of 8 toads and skins of 16 frogs (weighing 15—34 g, mean 23 g),

Flounder (*Pleuronectes flesus* L.)[1] skins of 3 flounders (weighing over 500 g).

Burbot (*Lota vulgaris* Jenyns)[1] skin of one burbot (weight 1.7 kg).

Pike (*Esox lucius* L.)[1] skins of 2 pikes (weighing 1—2 kg).

Smooth dogfish (*Mustelus mustelus* (L.))[2] skins of 4 fishes (lengths 40—50 cm). The skins were preserved about one week in 15 % ethanol at 4° before final treatment (Pikkarainen and Kulonen 1966),

Rayfish (*Raja* sp.)[3] abdominal skins of 6 rayfishes (lengths over 40 cm, widths over 40 cm).

Hagfish (*Myxine glutinosa* L.)[3] skins of 30 hagfishes (lengths 25—30 cm).

Lamprey (*Petromyzon fluviatilis* L.)[4] skins of 35 lampreys (lengths over 35 cm).

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[1] From the Turku archipelago in Finland.

[2] From the Mediterranean at the Marine Biological Station of Parma University at St. Margherita Ligure (courtesy of Fisherman Andrea Chiardello).

[3] From the Skagerrak at Kristineberg Zoological Station on the west coast of Sweden (courtesy of Dr. Bertil Swedmark).

[4] From the Pori archipelago in Finland (courtesy of Fisherman Erkki Lundgren).

Treatment of skins The animals were skinned immediately after slaughter the hagfishes and lampreys while still alive The hair, scales and subcutaneous fat were removed carefully The skins were rinsed with water cut into pieces and homogenized The frozen mammalian skins were ground in an ice cold meat grinder before homogenization The material was homogenized in a rotating blade disintegrator (Edmund Buhler, Tubingen, West Germany 50 000 rev /min)

Dry matter, ash content and hydroxyproline determinations were carried out on the rinsed material

FRACTIONATION OF COLLAGEN BY SOLVENT EXTRACTION

The isolation and purification of the various collagen fractions were performed at 4° unless otherwise stated All extractions were carried out overnight with agitation in a swirling shaker (C Desaga, GmbH, Heidel berg West Germany 80—140 strokes per minute) In the centrifugations either an MSF High Speed 17 or an MSE Superspeed 50 S refriger ated centrifuge (Measuring & Scientific Equipment Ltd London England) was used

Isolation and purification of neutral salt soluble collagen

The isolation of NSC from guinea pig and lamprey skins was carried out by repeated extraction with 0 45 M sodium chloride solution as de scribed in Fig 1 The neutral salt soluble collagen in combined extracts was purified according to Gross (1958a) as rapidly as possible The total recoveries were 74 2 % and 79 9 % respectively for guinea pig and lamprey collagens As a criterion of the purity the ratio of hydroxyproline nitrogen to total nitrogen was determined The ratios for NSC of guinea pig and lamprey were 0 082 and 0 071

Isolation and purification of citrate soluble collagen

Citrate soluble collagen was isolated by repeated extraction with 0 15 M citrate buffer of pH 3 7 of the tissue mass remaining after the extractions with sodium chloride solutions as described in Fig 1 The combined citrate extracts were purified according to the scheme in Fig 2

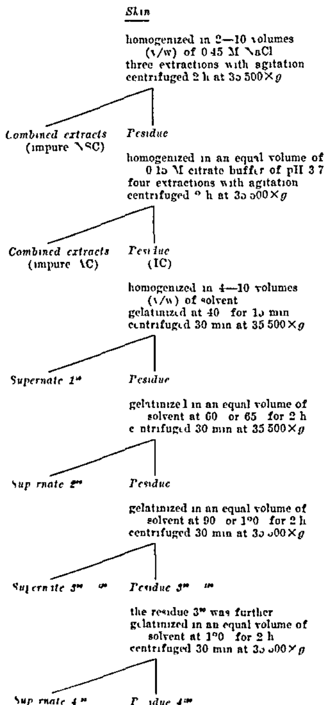


Fig. 1 Fractionation scheme for skin collagen

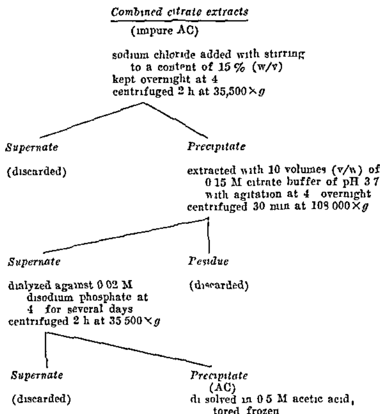


Fig 2 Purification scheme for acid soluble collagen

The recoveries of citrate soluble collagen after precipitation with sodium chloride and dialysis against disodium phosphate solution were 97 7 % and 96 9 % The ratio of hydroxyproline nitrogen to total nitrogen varied from 0 063 to 0 112

Isolation of insoluble collagen

After the citrate extractions the tissue mass was suspended in a *gelatinization medium agitated at 4° and centrifuged This was repeated* until the extract no longer contained protein (biuret reaction) The residue was gelatinized as shown in Fig 1

When the gelatinization was carried out in a 0 01 M sodium acetate buffer solution of pH 4 8 the ratio of hydroxyproline nitrogen to total nitrogen varied from 0 052 to 0 089 in supernates 1^{40} and from 0 046 to 0 098 in supernates 2° ^{65}

GENERAL ANALYTICAL METHODS

Dry matter and ash

A known weight of wet skin (0.1—5.0 g) was dried in a quartz crucible to constant weight (usually two days) at 95°. Dry matter determinations were carried out at least in triplicate. The coefficient of variation (100× standard deviation/arithmetic mean) of the method was ±5.1 %.

The dry matter was combusted at 530° in a muffle furnace (W. C. Heraeus, GmbH, Hanau, West Germany, Type MB 170) overnight. Weighing was always done after a constant period of time in a desiccator. The coefficient of variation in the determination of the ash content was ±10.8 %.

Hydroxyproline

The samples were hydrolysed in 5.7 N hydrochloric acid in tubes (culture tubes with screw caps and PTFE packings Kimax®) at 130° for 3 hours. The hydrochloric acid was volatilized on a boiling water bath. The dry residue was dissolved in distilled water immediately before the colour reaction was carried out by the method of Stegemann (1958) as modified by Woessner (1961).

A standard gelatin (acid processed pig skin gelatin No. 149 (see Biochem. J. 1961, 79, 652—656) a gift from Dr. J. E. Eastoe) was included in every series. The coefficient of variation of the method was ±2.2 %.

Nitrogen

The combustions were carried out by the method of Stegemann (personal communication to Dr. A. Minto). Half a millilitre of 8 N sulphuric acid was added to a sample containing 5—30 μg of nitrogen and the test tube was transferred to a hole in an aluminium block. The temperature of the block was raised to 300° on an electric plate and the samples were combusted until no organic material remained.

Then 3.0 ml of water was added to each tube followed by 3.0 ml of Nessler's reagent (made 0.015 M in potassium cyanide to prevent precipitation as proposed by Minari and Zilversmit (1963)). The absorbance was measured at 420 nm against a reagent blank after 30 min.

A series of ammonium sulphate standards was included in every digestion series. The coefficient of variation of the method was ±1.7 %.

α Amino nitrogen

The α amino nitrogen contents were determined according to Szentirmai *et al* (1962) Six glutamic acid standards of varying concentration were included in every series The coefficient of variation of the method was ± 6 9 %

Protein

The biuret method was a modification of that described by Lowry *et al* (1951)

A known volume, 0 030—2 00 ml of solution containing 0 005—0 2 mg of protein was pipetted into a test tube After the addition of 3 0 ml of freshly prepared alkaline copper reagent (50 volumes of 6 % sodium carbonate in 0 25 N sodium hydroxide solution and one volume of 0 5 % copper sulphate in 1 % sodium or potassium tartrate solution), the contents were mixed and left to stand for 10 min Diluted Folin s reagent (0 6 ml) was added and the mixture shaken immediately The absorbance at 750 nm was measured against the reagent blank after 30 minutes

Standard curves were made for the various sample volumes using standard gelatin (see the determination of hydroxyproline) The coefficient of variation of the method was ± 3 1 %

CONTENT OF COLLAGEN

The collagen content was calculated either from the content of hydroxyproline (by multiplying the hydroxyproline content by 7 3) or from the content of total nitrogen (by multiplying the nitrogen content by 5 55) or determined by the biuret method

DETERMINATION OF AMINO ACID COMPOSITION

Hydrolysis Protein (1—3 mg) was hydrolysed in 2—4 ml of 5 7 N hydrochloric acid under nitrogen at 110° for 20 hours After the hydrochloric acid had been volatilized on a boiling water bath the dry residue was dissolved in 0 2 N citrate buffer of pH 2 2 and stored frozen The α amino nitrogen content of the hydrolysate was determined

Description of the equipment and method Amino acid analyses were carried out by ion exchange chromatography with an automatic amino acid analyser built according to Spackman, Stein and Moore (1958)

Ground Amberlite resin CG 120 Type III 400 mesh was fractionated by the hydraulic method of Hamilton (1958) Fractions of particle sizes 68 ± 18 μ and 47 ± 11 μ were used to resolve neutral and basic amino acids, respectively

The buffer solutions were prepared exactly as proposed by Moore Spackman and Stein (1958) except that the pH of the first buffer solution used to elute the 150 cm column was $3\ 05 \pm 0\ 02$ instead of 3 25

The solvents were pumped through the 150 cm column with an Accu Flo® pump (Beckman Instruments Inc, Spinco Division Palo Alto, Calif, U S A) and through the 50 cm column in the early stages of the investigation with a MiniFlow precision micropump (LKB Produkter AB, Stockholm Sweden Type 4501) at a flow rate of 22 4 ml/h The ninhydrin reagent was also pumped with a MiniFlow pump at a rate of 11 2 ml/h Later the MiniFlow pumps were replaced by Accu Flo® pumps and the flow rates were increased to 40 ml/h and 20 ml/h respectively

The mixture of column effluent and ninhydrin reagent was led through PTFE tubing bore initially 0 5 mm and later 0 8 mm when the flow rate was increased through a boiling water bath where the mixture remained for at least 15 minutes The colorimeter was constructed precisely according to the instructions of Spackman *et al* (1958) Signals were registered with a multichannel recorder (Philips Type PR 3210 A/00), using a chart speed of 40 mm/h with a 1 mm interval between two successive printings on the same channel A special kind of logarithmic chart paper manufactured by A Ahlstrom Oy Fulan Paperi Kauttua Finland was used

The separation of acidic and neutral amino acids was carried out in *a* $150 \times 0\ 9$ *cm resin column at* $50 \pm 1°$ *The initial eluting solution was* a 0 20 N citrate buffer of pH $3\ 05 \pm 0\ 02$ A change to 0 20 N citrate buffer of pH $4\ 25 \pm 0\ 02$ was performed automatically so that cystine and valine were eluted by the second buffer A $50 \times 0\ 9$ cm resin column was used in the analysis of basic amino acids The eluting buffer was a 0 38 N citrate buffer of pH $4\ 26 \pm 0\ 02$ When lysine had emerged from the column the temperature was raised automatically from 30° to 50°

Standardization and calculation of results Norleucine (L Light & Co Ltd Poyle Colnbrook Bucks England) and β 2 thienylalanine (Nutritional Biochemical Corporation Cleveland Ohio U S A) were included as *internal standards in every chromatographic run through the 150 cm* column and β 2 thienylalanine and 2 amino 3 guanidopropionic acid (Cal

biochem Los Angeles Calif U S A) in every run through the 50 cm column (Siegel and Roach 1961, Walsh and Brown 1962)

For quantitation of the chromatographic peaks a standard mixture of amino acids (Amino Acid Calibration Mixture Type I for Amino Acid Analyzer, Lots CM 115 and CM 116, Beckman Instruments Inc, Spinco Division Palo Alto Calif U S A) was repeatedly chromatographed The following standard amino acids which were found to be pure by two dimensional paper chromatography were added to this mixture cysteic acid (L Light & Co Ltd Poyle Colnbrook Bucks England), methionine sulphoxide hydroxyproline (Fluka AG Buchs SG, Switzerland), delta hydroxylysine (K & K Laboratories Inc, Plainview New York U S A) and ornithine (S A F Hoffmann La Roche & Co AG Basle Switzerland)

The peak areas were calculated by multiplying the height (absorbance) of each peak by its width at half height A colour constant was obtained by dividing the number of micromoles of an amino acid by the respective peak area Relative colour constants were calculated for every amino acid by taking the constant for norleucine to be unity The amount of each amino acid in micromoles in the sample was obtained from the formulas

150 cm column	*50 cm column*
$X = q_x a_x/a$	$X = q_x a_x y/a_\beta q_\beta$

X = number of micromoles of amino acid x

q_x = relative colour constant of amino acid x (norleucine = 1 00)

q_β = relative colour constant of β 2 thienylalanine (norleucine = 1 00)

a_x = measured peak area of amino acid x

a = measured peak area of 1 00 μmole of norleucine

a_β = measured peak area of 1 00 μmole of β 2 thienylalanine

y = the ratio of the α amino nitrogen content of the sample added to the 150 cm column to the α amino nitrogen content of the sample added to the 50 cm column

The amino acid composition was expressed by giving the number of residues of the individual amino acids per 1000 residues

The mean coefficients of variation of the relative colour constants were ± 3 4 % (range 0 8—6 0 %) at 570 nm and ± 14 2 % (range 12 0—16 3 %) at 440 nm when using the 150 cm column and ± 6 1 % (range 4 9—8 4 %) when using the 50 cm column

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The mixture of column effluent and ninhydrin reagent was led through PTFE tubing bore initially 0.5 mm and later 0.8 mm when the flow rate was increased through a boiling water bath where the mixture remained for at least 15 minutes. The colorimeter was constructed precisely according to the instructions of Spackman *et al.* (1958). Signals were registered with a multichannel recorder (Philips Type PR 3210 A/00) using a chart speed of 40 mm/h with a 1 mm interval between two successive printings on the same channel. A special kind of logarithmic chart paper manufactured by A. Ahlström Oy Tutan Paperi Kauttua Finland was used.

The separation of acidic and neutral amino acids was carried out in a 150 × 0.9 cm resin column at 50 ± 1°. The initial eluting solution was a 0.20 N citrate buffer of pH 3.05 ± 0.02. A change to 0.20 N citrate buffer of pH 4.25 ± 0.02 was performed automatically so that cystine and valine were eluted by the second buffer. A 50 × 0.9 cm resin column was used in the analysis of basic amino acids. The eluting buffer was a 0.38 N citrate buffer of pH 4.26 ± 0.02. When lysine had emerged from the column the temperature was raised automatically from 30° to 50°.

Standardization and calculation of results. Norleucine (L. Light & Co. Ltd. Poyle, Colnbrook, Bucks. England) and β-2-thienylalanine (Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.) were included as internal standards in every chromatographic run through the 150 cm column and β-2-thienylalanine and 2-amino-3-guanidopropionic acid (Cal

position and rotations due to interactions within the helix and dependent upon the environment

K = constant that is a function of the mean residue weight of collagen and of the refractive index of the solvent

The equation was solved graphically by determining the wavelength λ_0 which gave a linear plot when $[\alpha]_\lambda(\lambda - \lambda_0)$ was plotted against $(\lambda - \lambda_0^2)^{-1}$

The mean values of λ_0 were found to be 251 nm and 254 nm for the studied native and denatured collagens respectively (Table VIII) The value 250 nm of λ_0 was used in the calculations because a variation of ± 5 nm is permitted by Moffitt and Yang in the choice of λ_0 and since it is essential to use the same value of λ_0 for both native and denatured macromolecules when determining the helix content (Urnes and Doty 1961) The constants Kb_0 and Ka_0 were obtained by dividing the graphically determined slope of the plot by λ_0^4 and the intercept by λ_0, respectively (Fasman 1963)

For the comparison of the helix contents of the various collagens a quantity related only to the helix content was formed as follows The obtained values of constants (Kb_0^D and Ka_0^D) for denatured collagen were subtracted from those (Kb_0^N and Ka_0^N) for native collagen This gives two terms both dependent on the helix content $Kb_0^N - Kb_0^D$ (written $Kb_0^{N\,D}$) and $Ka_0^N - Ka_0^D$ (written $Ka_0^{N\,D}$) which could be combined into one ($b_0^{N\,D}/a_0^{N\,D}$) by dividing the former by the latter This procedure is based on the assumption that the value of the constant K is the same for both native and denatured collagens Actually the value of K varies with temperature since it includes the refractive index which is known to be temperature dependent When however the optical rotatory dispersions are determined under comparable conditions the error due to the variation of K is the same in all measurements and can be neglected

DETERMINATION OF THE DENATURATION TEMPERATURE OF DISSOLVED COLLAGEN

Samples The denaturation temperatures of collagen solutions were determined on the same samples as were used for the determination of the optical rotations of native collagens

Equipment and procedure The polarimeter and the jacketed tube were those described on p 26 The jacket was connected to a water bath

and the circulation both in the bath and through the tube was maintained by two pumps (H. Heidolph, Schwabach, West Germany, Type P 50, 2700 rev./min). A coil through which cold tap water passed was immersed in the bath to minimize temperature fluctuations.

The optical rotation of a collagen solution was first measured at 365 nm against a 0.15 M citrate buffer of pH 3.7 at 8° (at 4° in the case of rayfish collagen). The temperature was then raised two degrees in two minutes and five minutes after the new temperature was reached, the temperature was measured again. The temperature was raised again (two degrees in two minutes) exactly after ten minutes had elapsed from the beginning of the preceding rise and the rotation was measured seven minutes after the temperature had been raised. This procedure was repeated until the rotation no longer changed.

Calculations. The specific rotations were calculated and the denaturation curve was constructed by plotting $([\alpha]^{t}_{365} - [\alpha]^{D}_{365})/([\alpha]^{N}_{365} - [\alpha]^{D}_{365})$ as a function of temperature.

$[\alpha]^{t}_{365}$ = specific rotation at 365 nm and temperature t

$[\alpha]^{D}_{365}$ = specific rotation of completely denatured collagen at 365 nm

$[\alpha]^{N}_{365}$ = specific rotation of native collagen at 365 nm

Because of the dependence of the optical rotation on the secondary structure of collagen, this ratio gives the helix content at a given temperature. The denaturation temperature (T_D) of a collagen solution was defined as the temperature where a 50 % change in optical rotation had occurred.

DETERMINATION OF THE SHRINKAGE TEMPERATURE OF SKIN

Samples. The hair, feathers or scales and the subcutaneous fat were removed from the skins which were then rinsed in running tap water and stored frozen until analysed. Before analysis, the thawed specimens were kept in distilled water at 4° which was replaced repeatedly for at least 24 hours. The skin of the smooth dogfish was an exception, for it was preserved in 15 % aqueous ethanol at 4° about one week before rinsing (Pikkarainen and Kulonen 1966).

Equipment and measurements. A piece of skin 1 × 5 cm with punched holes in each end (Fig. 3) was hung with the aid of two hooks from a rubber stopper (I). A 60—500 mg stainless steel weight (G) was attached

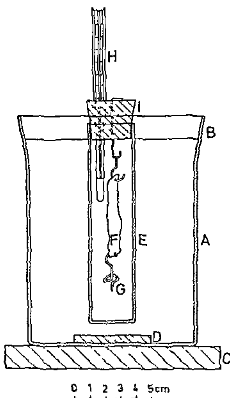


Fig 3 The apparatus used in the determination of the thermal shrinkage temperature
A water filled glass vessel *B* water level *C* heating plate and magnetic stirrer *D* PTFE coated magnetic rod *E* water filled glass tube *F* 1×5 cm skin strip *G* stainless steel weight *H* thermometer and *I* rubber stopper

to the lower end of the skin strip Additional weights up to 20 g could be placed on the first weight The loaded skin strip was first submerged in cold water in a glass tube (E) which was immersed in cold water in another glass vessel (A) located on the heating plate (C) of a magnetic stirrer (IKA Combimag® RCH, Jahnke & Kunkel KG Staufen i Breisgau Germany 250/500 W, 80—800 rev /min) The temperature was recorded close to the skin strip in the inner tube (E) with a mercury thermometer (H) (0—100° subdivisions 1°)

The length of skin strip between the holes and the position of the lower end of weight were recorded with a cathetometer (W G Pye & Co Ltd Cambridge England subdivisions 0 005 mm) at 5° The temperature in the inner tube was raised one degree per minute and the position of the weighted end as well as the temperature were recorded at one minute intervals until a temperature of 95° was reached

The relative change in the length of the skin strip was plotted as a function of temperature The shrinkage temperature (T_s) is defined as the temperature where the greatest shortening of the skin strip per degree occurred

FRACTIONATION OF COLLAGEN COMPONENTS BY STARCH GEL ELECTROPHORESIS

Samples The samples of collagen were either dialyzed against or dissolved in a sodium acetate buffer of pH 4 7 (I = 0 017) to give an about 0 5 % solution Before fractionation the sample solution was denatured by keeping it at least 15 minutes at 40°

Method The starch gel electrophoresis of collagen has been described in detail earlier (Nanto *et al* 1963 1965) Eleven grams of starch (Connaught Medical Laboratories, Ltd Toronto Canada) suspended in 75 ml of a sodium acetate buffer solution was heated in a flask over a flame with continuous stirring When the formed gel began to boil it was deaerated with a water pump half a minute The gel was poured into a trough covered with a glass plate and allowed to cool at least 1 5 hours It was rewarmed to 38° before application of the denatured collagen sample solution imbibed in a piece of Whatman 3 MM filter paper The moist filter paper was inserted in a perpendicular slot in the anode end of the gel and the gel sheet was covered with a thin film of plastic before applying the current

The combined volume of the buffer compartments was 3 5 l The voltage applied to the electrodes was 125 V, and the duration of electrophoresis was usually six hours The electrophoresis was carried out in a room held at 38°

The stained and washed gels were photographed on Adox KB 14 or Agfa Agepan films using a dark red filter (Johannes Weber KG, Wiesbaden West Germany) for better contrast The absolute and relative migrations were calculated for the various components In the calculation of relative migration the distance travelled by the fastest moving band in each gel was taken as 100

Standardization An attempt was made to keep the voltage constant during the run by manual adjustment In order to get comparable electrophoretic migration data for various collagens purified citrate soluble collagen of guinea pig skin was used as an internal standard in every electrophoretic run in each gel sheet

When the effect of ionic strength or pH on the electrophoretic pattern was studied all the respective runs were carried out simultaneously The conductances of the buffers used were also measured

FRACTIONATION OF COLLAGEN COMPONENTS BY CARBOXY METHYLCELLULOSE COLUMN CHROMATOGRAPHY

Samples. The samples of citrate soluble collagens were dialyzed against 0.01 M sodium acetate buffer of pH 4.7 at 4°. Before chromatography the dialyzed solutions were denatured by keeping them at least 15 minutes at 40°.

Equipment and procedure. A jacketed chromatographic tube (inside diameter 20 mm) held at 38° was packed under nitrogen pressure to a column height of 30 cm with CM cellulose (Whatman CM 70 or Whatman Chromedia grade CM 11) that had been preswollen in the starting buffer at least one night. The packed column was then equilibrated with the starting buffer.

A linear gradient (Piez *et al.* 1963) between the starting and limiting buffers was obtained with a peristaltic pump (C. Desaga, Heidelberg, West Germany) as described by Davis, Santen and Agranoff (1965). The limiting buffer was prepared by adding sodium chloride to the starting buffer, a sodium acetate buffer of pH 4.8 with I = 0.03, 0.04 or 0.06, until the sodium chloride concentration was 0.10 molar (I = 0.13, 0.14 or 0.16). Sometimes the column was eluted further with 1.7 M sodium chloride solution applied directly onto the bed after the gradient. The effluent was collected in 2 or 4 ml fractions in a collector (Dr. Hans Hoshi, Bischofszell, Switzerland, Type TVZ 260) at a constant flow rate of 60 ml/h. All connections to and from the column were small bore (1.0—1.5 mm) polythene tubing. The buffers were deaerated by heating and the liquid in the mixing vessel was covered with liquid paraffin.

Protein in the effluent was detected either with the biuret reaction or before the collection of the fractions, by recording the transmittance at 230 nm with a UV—Vis 139 Hitachi—Perkin—Elmer spectrophotometer equipped with a photomultiplier, a flow cell (light path 5 mm) and a Hitachi—Perkin—Elmer Type 159 recorder.

Combined effluent fractions were freeze dried to reduce the volume and desalted on Sephadex G 25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) columns at 38° using pyridine acetate buffer of pH 4.7 (0.5 % pyridine in 0.45 % acetic acid) for elution. The desalted solutions were freeze dried again and the residue was dissolved with slight warming when necessary in either 0.5 M acetic acid or the sodium acetate buffer of pH 4.7 (I = 0.017) used in the starch gel electrophoresis. The fractions were stored at —20°.

RESULTS

AMINO ACID COMPOSITIONS

The amino acid compositions of the citrate soluble collagens of several vertebrates are presented in Tables VI and VII. The results confirm the known constancy of the content of nonpolar amino acids and the sum of the contents of acidic and hydroxy amino acids as well as the absence of cystine (Tables I and II). The content of glycine is about one third of the total amino acid content in different collagens. Not only the glycine content but also the contents of alanine, glutamic acid and aspartic acid vary only slightly.

Pig and chick collagens have high contents of both imino acids but low contents of serine and threonine compared to collagens of fishes and cyclostomes. The methionine contents are also lower in the pig and chick collagens than in the collagens of fishes, except flounder.

The collagens of the cartilaginous fishes, dogfish and rayfish, have higher contents of isoleucine and arginine (and hence basic amino acids) than the collagens of all the other species examined in the present study and contain more glutamic acid, valine and leucine than flounder or cyclostomes. The amino acid compositions of dogfish and rayfish collagens seem to differ only in the serine, threonine and imino acid contents that are believed to be responsible for the stability of collagen.

The collagens of cyclostomes, hagfish and lamprey, have lower contents of leucine than the other collagens studied. Lamprey collagen resembles the collagens of higher vertebrates more than hagfish collagen in having a higher content of imino acids and less serine and threonine.

An evolutionary trend in the amino acid composition of collagen can be observed in the contents of imino acids, threonine, serine, methionine and histidine. Flounder collagen is not typical as in the contents of threonine, methionine and histidine it resembles more the collagens of higher vertebrates than the collagens of fishes. The composition of lamprey collagen also differs from the expected in the contents of various amino acids (see Discussion).

Table VI

Amino acid compositions of citrate soluble collagens

The values are numbers of amino acid residues per 1000 residues and have not been corrected for losses during hydrolysis and chromatography. The numbers in parentheses are the numbers of determinations

	Pig ([illegible])	Chi[illegible]	Frog	Flounder (2)	Dogfish ([illegible])	Rayfish	Hagfish (2)	Lamprey (2)
Hydroxyprolines (3 & 4)	91	99	65	63	72	61	62	66
Aspartic acid	46	4[illegible]	5[illegible]	47	42	42	50	49
Threonine	18	19	22	20	25	30	[illegible]7	23
Serine	35	2[illegible]	[illegible]6	6[illegible]	46	71	71	[illegible]8
Glutamic acid	7[illegible]	[illegible]3	6[illegible]	58	72	72	59	67
Proline	13[illegible]	113	102	103	99	84	92	107
Glycine	3[illegible]0	334	362	374	333	332	381	3[illegible]6
Alanine	111	127	12[illegible]	120	119	97	116	121
Half cystine (and cysteic acid)	—	—	—	—	—	—	—	—
Valine	26	19	17	18	25	[illegible]3	17	17
Methionine (and sulphoxides)	4	7	5	7	11	14	11	10
Isoleucine	10	12	11	7	18	19	10	8
Leucine	24	26	25	20	27	28	16	19
Tyrosine	3	<0 5	2	1	<0 5	5	2	1
Phenylalanine	13	10	11	[illegible]	10	14	8	9
Hydroxylysines	7	3	3	3	3	3	3	4
Ornithine	—	1	—	—	—	—	—	<0 5
Lysine	[illegible]6	27	[illegible]6	26	29	26	14	23
Histidine	4	3	3	4	7	10	7	6
Arginine	49	53	51	57	62	69	54	56
Acidic amino acids	118	1[illegible]0	117	10[illegible]	114	114	109	116
Basic amino acids	86	87	83	90	101	108	[illegible]	89
Difference	3[illegible]	33	34	1[illegible]	13	6	31	2[illegible]
Imino acids	2[illegible]3	21[illegible]	167	166	171	145	154	1[illegible]3
Hydroxy amino acids	154	148	148	15[illegible]	146	1[illegible]0	165	152
Nonpolar amino acids	6[illegible]0	648	655	6[illegible]6	642	611	6[illegible]1	647

Pig skin gelatin see p 2[illegible]

Table VII

Variations in the amino acid contents of citrate soluble collagens

The values are based on the data in Table VI

	Range	Δ	Mean	Δ/Mean
Hydroxyprolines (3 & 4)	61— 99	38	80	0.48
Aspartic acid	42— 55	13	48.5	0.27
Threonine	18— 30	12	24	0.50
Serine	27— 71	44	49	0.90
Glutamic acid	58— 73	15	65.5	0.23
Proline	84—132	48	108	0.44
Glycine	330—381	51	355.5	0.14
Alanine	97—127	30	112	0.27
Half cystine (and cysteic acid)	—	—	—	—
Valine	17— 26	9	21.5	0.42
Methionine (and sulphoxides)	4— 14	10	9	1.11
Isoleucine	7— 19	12	13	0.92
Leucine	16— 28	12	22	0.55
Tyrosine	<0.5— 5	5	2.5	2.00
Phenylalanine	7— 14	7	10.5	0.67
Hydroxylysines	3— 7	4	5	0.80
Ornithine	0— 1	1	0.5	2.00
Lysine	14— 29	15	21.5	0.70
Histidine	3— 10	7	6.5	1.08
Arginine	49— 69	20	59	0.34
Acidic amino acids	105—120	15	112.5	0.13
Basic amino acids	78—108	30	93	0.32
Difference	6— 34			
Imino acids	145—223	78	184	0.4
Hydroxy amino acid	146—170	24	158	0.15
Nonpolar amino acids	611—656	45	633.5	0.07

OPTICAL ROTATORY DISPERSIONS

The optical rotations of several citrate soluble collagens were determined at different wavelengths to evaluate the optical rotatory dispersions. Two equations were fitted to the optical data. The Drude equation describes the rotatory dispersion in regions distant from absorption bands whereas the equation of Moffitt and Yang gives the helix content more reliably. The results of the calculations are presented in Table VIII.

Table VIII

Optical rotatory dispersion values of citrate soluble collagens

The value λ = 250 nm was used in the calculation of the values of Kb and Ka

The letters H and D refer to native and denatured collagens respectively

	Drude					Moffitt Yang						
	λ^H	λ^D	$A^H \times 10$	$A^D \times 10$	$(A^H - A^D) \times 10^r$	λ^H	λ^D	Kb^H	Kb^D	Ka^H	Ka^D	b^{HD}/a^{HD}
Calf	198	205	—13 19	—5 96	— 9 23	237	206	647	276	—2392	—918	—0 26
Guinea pig												
full grown	188	200	—17 00	—5 55	—11 45	240	240	801	257	—2603	—870	—0 32
growing	200	214	—1 99	—4 71	— 9 28	248	262	519	150	—2029	— 42	—0 29
Chick	201	210	—13 53	—4 88	— 8 65	245	25	540	166	—2122	—670	—0 28
Frog	212	204	—11 07	—4 32	— 6 75	2 2	2 5	366	169	—1746	—690	—0 18
Flounder	202	195	—13 49	—3 98	— 9 51	257	2 7	594	166	—2110	—618	—0 24
Burbot	202	230	—13 0	—3 26	— 9 76	249	276	519	84	—2046	—5 8	—0 29
Pike	03	206	—12 49	—4 23	— 8 26	248	251	491	150	—1963	—601	—0 26
Dogfish	202	214	—13 10	—4 04	— 9 06	245	265	517	133	—2054	—640	—0 27
Rayfish	200	203	—11 0	—3 44	— 7 76	242	250	470	156	—1766	—536	—0 28
Hagfish	01	215	—10 30	—3 71	— 6 59	262	262	376	119	—1600	—587	—0 25
Lamprey	203	201	—12 76	—4 33	— 8 43	250	240	501	167	—2006	—675	—0 25
Mean	201	208	—13 01	—4 37	— 8 64	251	254	523	161	—2041	—610	—0 26

The rotatory dispersions of the investigated collagens are of the simple type over the studied region 365—578 nm and can hence be represented by the one term Drude equation The values of λ for both native (^{n}) and denatured (^{D}) collagens are nearly equal, mean values 201 nm and 208 nm and independent of the species Since the constant A varies with the helix content of proteins the difference A^{N}—A^{D} is an estimate of the helix content and seems to be relatively invariable in the collagens studied

When the obtained dispersion values were substituted in the Moffitt Yang equation the mean values of λ_0 were 251 nm for the native and 254 nm for the denatured collagens For the comparison of helix contents the quantity $b^{N\,D}/a^{N\,D}$ was calculated from the values of constants obtained for native (Kb_0^N and Ka^N) and denatured (Kb_0^D and Ka_0^D) collagens Table VIII shows that also the values of this quantity for the various collagens are nearly equal

To recapitulate the results of the studies on optical rotatory dispersion the values of λ obtained for both native and denatured collagens are nearly equal for all collagens studied regardless of species The same applies to λ_0 The helix content of collagen does not seem to vary from one species to another

DENATURATION TEMPERATURES

The denaturation temperatures of dissolved collagens give information only on the intramolecular forces that stabilize the tropocollagen molecule The thermal shrinkage temperatures give in addition information on the interaction of native tropocollagen molecules

Table IX presents values of T_D for several citrate soluble collagens (T_D is defined as the temperature at which a 50 % change has occurred in optical rotation) Mammalian and avian collagens that is, collagens of homoiothermic animals have T_D values at least 15° higher than the

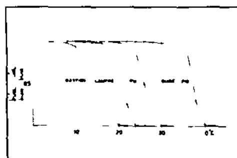


Fig 4 Denaturation curves of different citrate-soluble collagens
Abscissa temperature *Ordinate* the proportion of native collagen at temperature t determined from optical rotation

Table IX

Denaturation temperatures of collagens in the liquid (T_D) and solid (T_S) state

The T_D values were determined by polarimetry of solutions of citrate soluble collagens in a 0 15 M citrate buffer of pH 3 7 The T_S values and the relative contractions of the skins were determined in water

	T_D	T_S		Shrinkage percent	
		First	Second	First	Second
Cow	—	70 —73	—	—	—
Calf	37 4	68 —71	—	35—50	—
Guinea pig					
full grown	37 1	—	—	—	—
growing	37 3	—	—	—	—
Chick	40 7	64 —67	—	35—45	—
Frog	25 3	58 —61	—	30—35	—
Flounder	20 7	51 —53	(72 —76 ?)	35—45	(10—25?)
Burbot	19 8	—	—	—	—
Pike	6 0	—	—	—	—
Dogfish	25 1	53 —55	(65 —75 ?)	10—20?	(5—20?)
Rayfish	13 2	36 —39	60 —64	15—35	5—25
Hagfish	15 7	33 —36	59 —62	15—30	(—15)—25
Lamprey	21 0	44 —46	65 —68	3—6	20—25

T_D values of collagens of poikilothermic animals The denaturation curves in Fig 4 show that rayfish collagen begins to lose its ordered structure already at temperatures below 10°

The measured thermal shrinkage temperatures reveal that fish and cyclostome skins have distinctly lower T_S values than the skins of warm blooded animals (Table IX) Another remarkable property that places

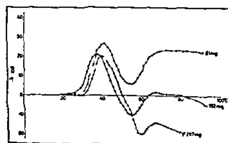


Fig 5 Biphasic thermal shrinkage curve of hagfish skin loaded with various weight
Abscissa temperature *Ordinate* shrinkage as a percentage of the original length (l) at temperature t

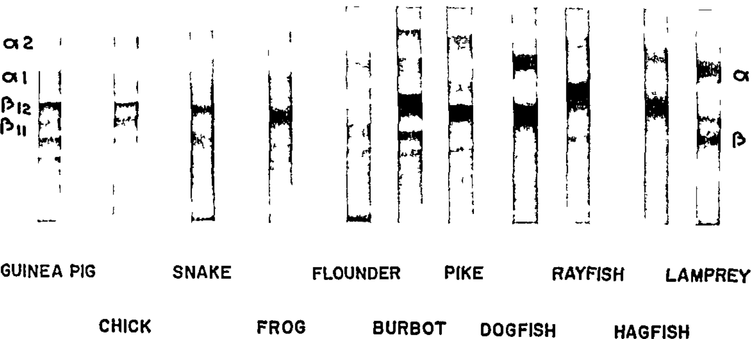


Fig 6 Starch gel electrophoretic patterns of citrate-soluble collagens from skins of vertebrates. Although there are two α components in the collagens of bony fishes, there is only one α component in the collagens of cyclostomes and cartilaginous fishes.

Electrophoretic conditions gel concentration 14.7 %, sodium acetate buffer of pH 4.7 (I = 0.017), 125 V for 6 h at 3°. Migration upwards.

these aquatic animals in a separate category is the biphasic form of the denaturation curves of their collagens as shown in Fig 5 Such a curve was obtained for all the studied fish and cyclostome skins except dogfish skin The extent of the first shrinkage varied from one species to another but was independent of the applied weight for one animal but the second shrinkage depended on the weight applied

The mean differences T_S (1st shrinkage)—T_D and T_S (2nd shrinkage)—T_D are 27° (range 19°—34°) and 48° (range 45°—53°) respectively

COMPONENTS RESOLVED BY STARCH GEL ELECTROPHORESIS

A general picture of starch gel electrophoretic patterns of citrate soluble collagens is provided by Fig 6 where the animals are arranged in phylogenetic order The figure shows that the electrophoretic pattern is more complex in higher animals and that the evolutionary time interval between cartilaginous and bony fishes means a juncture in the evolution of collagen flounder being an exception Although there are two α components in the collagens of bony fishes there is only one α component in the collagens of cyclostomes and cartilaginous fishes

In order to make sure of the nonidentity of the α chains guinea pig collagen was mixed with both hagfish and lamprey collagens and these mixtures were subjected to gel electrophoresis (Figs 7—8)

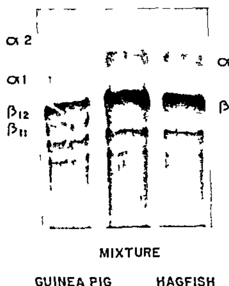
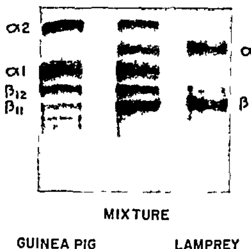


Fig 7 Starch gel electrophoresis of a mixture of citrate soluble skin collagens of hagfish and guinea pig

Electrophoretic conditions gel concentration 14 %, sodium acetate buffer of pH 4 7 (I = 0 01) 15 V for 6 h at 3°

Fig 8 Starch gel electrophoresis of a mixture of neutral salt soluble skin collagens of lamprey and guinea pig

Electrophoretic conditions gel concentration 14 7 %, sodium acetate buffer of pH 4 7 (I = 0 017), 125 V for 6 h at 38



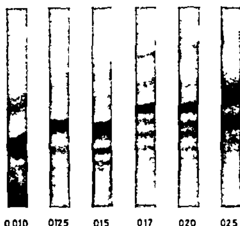


Fig 9

Figs 9—11 Starch gel electrophoretic patterns of citrate-soluble collagen of guinea pig skin when the ionic strength of the buffer was varied.

Fig 9 Photograph of original patterns

Fig 10 Schematic presentation of patterns in Fig 9

Abscissa ionic strength *Ordinate* electrophoretic mobility (cm²V sec⁻¹) The various hadings refer to the intensities of the individual bands as estimated visually

Fig 11 Like the previous figure but the *ordinate* is the relative mobility (the distance moved by the leading edge of the fastest band in each gel was taken as 100)

Electrophoretic conditions gel concentration 14 7 % sodium acetate buffer of pH 4 7 125 V for 6 h at 38



Fig 10

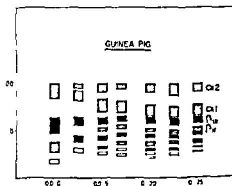


Fig 11

The behaviour of various components was studied at different ionic strengths keeping the other conditions unchanged Fig 9 presents a photograph of electrophoretic patterns from one series of such experiments For clarity this figure was redrawn and the following two figures (Figs 10—11) show two ways of presenting starch gel electrophoretic patterns

The citrate soluble collagens studied can be grouped into three classes (Fig 25) on the basis of the migration behaviour of their main components as follows The first class, the so called guinea pig type comprises guinea pig (Figs 9—11) chick (Fig 12), frog (Fig 13) burbot (Fig 14) and pike collagens (Fig 15) The α components and the β components of the

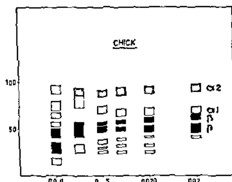


Fig 12



Fig 13

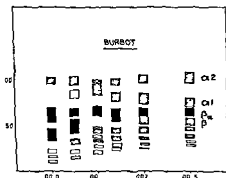


Fig 14

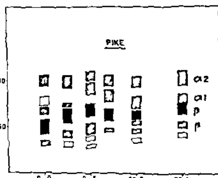


Fig 15

Figs 12—15 Effect of ionic strength on the starch gel electrophoretic patterns of citrate soluble skin collagens (guinea pig type) of vertebrates

The α-components and the β-components of the guinea pig type collagens are more effectively resolved when the ionic strength is increased

Abscissa ionic strength, *Ordinate* relative mobility

Electrophoretic conditions gel concentration 14 7 % sodium acetate buffer of pH 4 7, 1?5 V for 6 h at 38

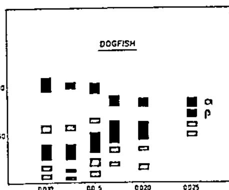


Fig 16

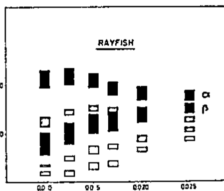


Fig 17

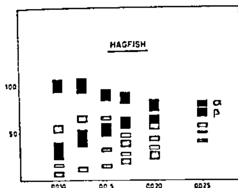


Fig 18

Figs 16—18 Effect of ionic strength on the starch gel electrophoretic patterns of citrate soluble skin collagens (rayfish type) of vertebrates

The mobilities of the α and β components of the rayfish type collagens approach each other when the ionic strength is increased

Abscissa ionic strength *Ordinate* relative mobility

Electrophoretic conditions gel concentration 14 7 % sodium acetate buffer of pH 4 7 125 V for 6 h at 38

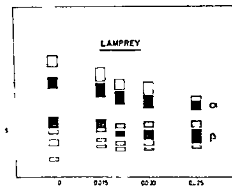


Fig 19

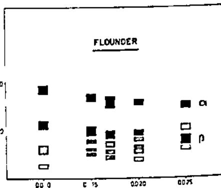


Fig 20

Figs 19—20 Effect of ionic strength on the starch gel electrophoretic patterns of citrate soluble skin collagens (lamprey type) of vertebrates

The mobilities of the α and β-components of lamprey and flounder collagens vary similarly with ionic strength

Abscissa ionic strength *Ordinate* relative mobility

Electrophoretic conditions gel concentration 14 7 % sodium acetate buffer of pH 4 7, 125 V for 6 h at 38

guinea pig type collagens are more effectively resolved when the ionic strength is increased The second class (the rayfish type) comprises dog fish (Fig 16), rayfish (Fig 17) and hagfish collagens (Fig 18) The mobilities of the α and β components of these collagens approach each other when the ionic strength is increased The third type the lamprey type includes flounder collagen (Fig 20) in addition to lamprey collagen (Fig 19) The mobilities of the α and β components of these collagens vary similarly with ionic strength

The effect of pH on the electrophoretic patterns of the three types of collagen resembled the effect of ionic strength as seen in Figs 21—24 The results of these studies on the effect of ionic strength and pH are summarized schematically in Fig 25 The α and β components of rayfish and lamprey collagens were identified by sedimentation analyses (carried

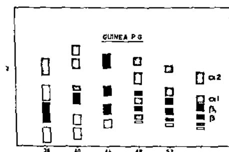


Fig 21

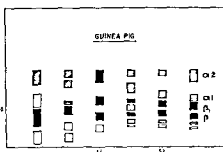


Fig 22

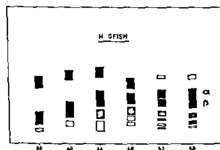


Fig 23

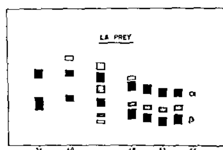


Fig 24

Figs 21—24 Effect of pH on the starch gel electrophoretic patterns of citrate soluble skin collagens of vertebrates

The effect of pH on the electrophoretic mobilities of the α and β-components of the various collagen types resembles the effect of ionic strength

Abscissa pH *Ordinate* relative mobility in Figs 22—24 and electrophoretic mobility (cm V c) in Fig 21

Electrophoretic conditions gel concentration 14 7 %, sodium acetate buffer (I = 0 01) 1 5 V for 6 h at 38

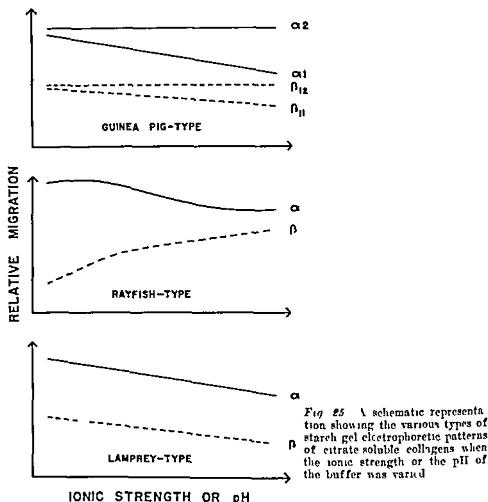


Fig 25 A schematic representation showing the various types of starch gel electrophoretic patterns of citrate soluble collagens when the ionic strength or the pH of the buffer was varied

out by Dr T Hollmén) which gave the sedimentation constant ($s^{0}_{20,w}$) 4 22S for the faster component and the sedimentation constant 3 04S for the slower component of rayfish collagen and the constants 4 14S and 3 17S for the faster and slower components respectively of lamprey collagen

The appearance of a second and a third new α component during evolution is discussed later (Fig 39)

A study of the effect of varying the gel concentration was carried out with guinea pig (Figs 26—27) and rayfish collagens (Figs 28—29) The sieve effect which separates the α and β components according to their molecular weights is observed with both collagens this separation is more marked at the lower ionic strength (0 010) The electrophoretic

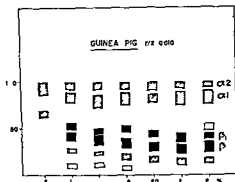


Fig 26

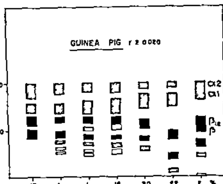


Fig 27

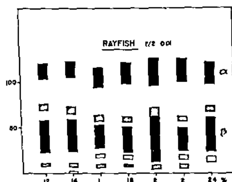


Fig 28

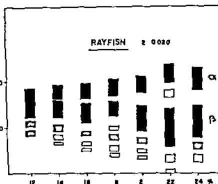


Fig 29

Figs 26—29 Effect of gel concentration on the starch gel electrophoretic patterns of citrate-soluble skin collagens of guinea pig and rayfish

Abscissa gel concentration (w/v) *Ordinate* relative mobility

Electrophoretic conditions sodium acetate buffer of pH 4 7 (I = 0 010 in Figs 26 and 28 I = 0 020 in Figs 27 and 29) 150 V for 6 h at 3°

mobilities of the components decreased with increasing gel concentration but the changes in the relative mobilities differed from those observed when the ionic strength or pH was altered

COMPONENTS RESOLVED BY CARBOXYMETHYLCELLULOSE COLUMN CHROMATOGRAPHY AND STARCH GEL ELECTROPHORESIS

The citrate soluble collagens of the different species studied could be divided into two groups on the basis of their patterns in CM cellulose chromatograms The first group comprised the guinea pig and rayfish types which all gave similar fractionation patterns composed of two major

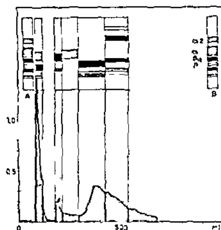


Fig 30

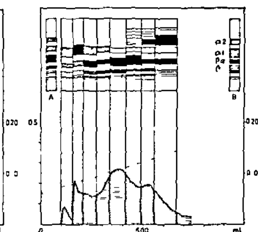


Fig 31

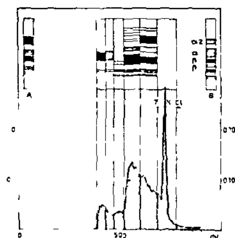


Fig 3

Figs 30—32 CM cellulose chromatography of citrate olubl skin collag ns (guinea pig type) of vertebrates and the tarch g l electrophoresis of chromatographi fraction

The α and β components of guinea pig type collagens are eluted from the CM cellulo e column in the ord r α1 β β and α

Abscis s effluent volume (ml) Ordinate optical density (left) and ionic strength (right) Solid line protein in the effluent detected with the biuret reaction (al orption at 530 nm in Fig 31) Dash d line ionic trength of eluting olution A tarch gel electrophor tic pattern before chromatography B starch gel electrophoretic pattern of citrat solubl guinea pig collagen Migration upward

peaks with houlders The collagens of lamprey and flounder formed the second group

The first group could be further divided into two subgroups, the guinea pig and rayfish types by subjecting the chromatographic fractions of collagens to starch gel electrophoresis The α and β components in the guinea pig type collagens (Figs 30—32) were eluted from the CM

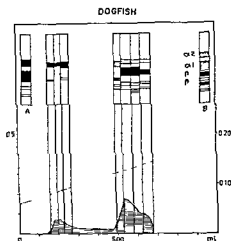


Fig 33

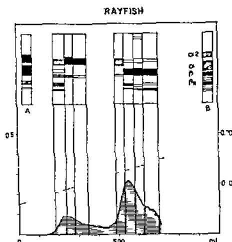


Fig 34

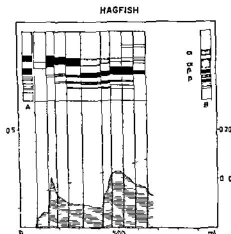


Fig 35

Figs 33—35 CM cellulose chromatography of citrate soluble skin collagens (rayfish type) of vertebrates and the starch gel electrophoresis of chromatographic fraction

The single α-component of the rayfish type collagens is eluted before the β component in CM cellulose chromatography

Abscissa effluent volume (ml) *Ordinate* optical density (left) and ionic strength (right) *Solid line* protein in the effluent (absorption at 230 nm) *Dashed line* ionic strength of eluting solution *A* starch gel electrophoretic pattern before chromatography *B* starch gel electrophoretic pattern of citrate soluble guinea pig collagen

cellulose column in the order $\alpha 1$, β_{11}, β_1 and $\alpha 2$ The CM cellulose pattern of the rayfish type collagen (Figs 33—35) differs from that of the guinea pig type in that the α component and the β component are more highly resolved In both types higher aggregates are eluted before the β_1 or β component The scheme in Fig 36 shows the behaviour of the α and β components of guinea pig type and rayfish type collagens in chromatography and gel electrophoresis

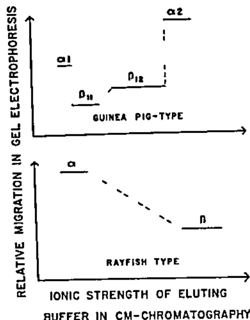


Fig. 36. A schematic representation showing the various types of behaviour of citrate soluble collagens of guinea pig and rayfish types in CM cellulose chromatography and subsequent starch gel electrophoresis.

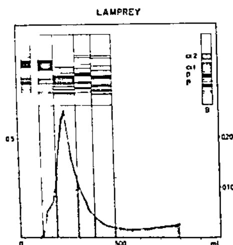


Fig. 37

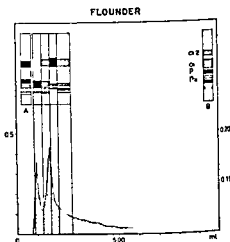


Fig. 38

Figs. 37–38. CM cellulose chromatography of citrate soluble skin collagens (lamprey type) of vertebrates and the starch gel electrophoresis of chromatographic fraction. *Abscissa*: effluent volume (ml). *Ordinate*: optical density (left) and ionic strength (right). *Solid line*: protein in the effluent (absorption at 230 nm). *Dashed line*: ionic strength of eluting solution. *A*: starch gel electrophoretic pattern before chromatography. *B*: starch gel electrophoretic pattern of citrate-soluble guinea pig collagen.

The lampiev and flounder citrate soluble collagens of the second group were completely eluted from the CM cellulose column by an eluting buffer of lower ionic strength than the other collagens The chromatographic separation of the α and β components of lamprey collagen (Fig 37) was poor, but otherwise this collagen resembled the rayfish type Flounder collagen (Fig 38) differed from lamprey collagen in being more effectively resolved in CM cellulose chromatography but the components were eluted in the reverse order No data are available but the two peaks of flounder collagen may represent components α1 and α2, and not an α component and a β component

SOLUBILITY OF NATIVE AND DENATURED COLLAGENS

Repeated extraction of finely homogenized skin with 0 45 M sodium chloride solution followed by 0 15 M citrate buffer of pH 3 7 at 4° resulted in the dissolution of soluble collagens as shown in Table X From 30 to

Table X
Ash hydroxyproline and soluble collagen contents of vertebrate skins

	Ash	Hydroxy proline *	Percent collagen soluble in	
			neutral salt solution	acid buffer solution
Cow	2 2	9 9	0 4	2 5
Calf	2.3	7 9	1 4	24 4
Guinea pig (full grown)	2 0	6 9	7 5	17 1
Chick	0 8	_ 3	18 0	6 7
Snake	4 5	4 2	0 5	1 3
Frog	6 3	4 6	? 7	4 5
Flounder	1 5	7 3	10 6	13 1
Burbot	0 9	7 9	1 8	46 8
Pike	0 6	7 6	1 2	33 [illegible]
Dogfish	26 9	8 4	1 9	44 0
Rayfish	3 7	6 2	0 4	89 5
Hagfish	0 9	8 4	0 5	78 3
Lamprey	0.8	5 6	2 1	74 6

Ash as a percentage of the weight of the dry skin
g/100 g of dry ash free skin

90 % of the skin collagens of cyclostomes and fishes but only a few percent of the collagens of a full grown mammal passed into the citrate solution A similar difference connected with age was noted on comparing the dissolution of collagens from the skins of a calf and a cow

The decrease of the solubility of collagen during evolution was reflected in the more facile gelatinization of insoluble cyclostome and fish skin collagens Table XI shows that more than half of their insoluble collagens dissolved already when the skin residues, after extraction of the soluble collagens, were warmed in 0 01 M sodium acetate buffer of pH 4 8 at 40° for 15 minutes The corresponding percentages for the insoluble collagens of mammals were less than 10

Table XI

Gelatinization of insoluble skin collagens from various vertebrates

Collagen solubilized in 0 01 M sodium acetate buffer of pH 4 8 at progressively raised temperatures is expressed as a percentage of the original insoluble collagen The symbols for the various extracts are explained in Fig 1

	S 1[40]	S 2[45]	S 3[50]	P 3[50]: S 4[60]	P 3[50]: P 4[60]
Cow	0.5	6.0	4.9	84.3	4.3
Calf	2.7	63.7	12.3	20.1	1.2
Guinea pig					
full grown	9.[illegible]	60.6	14.3	15.4	0.5
growing	4.2	85.4	[illegible]8	3.9	0.7
Chick	6.0	59.1	20.4	[illegible]1.5	
Snake	2.7	[illegible]3	[illegible]0	6[illegible].0	
Toad	16.9	38.1	35.6	9.4	
Burbot	60.3	12.6	11.5	6.6	
Pike	8.4	13.5	4.2	3.9	
Hagfish	55.0	16.1	10.8	17.[illegible]	
Lamprey	80.0	4.4	3.1	2.6	

Gelatinized hours at +60

DISCUSSION

GENERAL

Collagen is a very old protein 550 million years old or perhaps older as judged from its wide distribution in the animal kingdom The information available on the evolution of collagen obtained from studies of fossils is rather meagre and provides only the age of collagen It has been established by electron microscopy and X ray diffraction methods that the oldest collagen fibrils in remnants of bones and teeth are 170—200 million years old (Isaacs *et al* 1963 Doberenz and Lund 1966) From the amino acid compositions of residues from fossils, Abelson (1963) and Armstrong and Tarlo (1966) *concluded that collagen existed in animals that lived* 370 million years ago

Since organic material easily decomposes with time fossils are not suitable for investigations of the evolution of collagen Knowledge of the phylogeny of collagen can thus be only derived from existing animals by methods of comparative biochemistry

PRIMARY STRUCTURE

Because the complete amino acid sequences of collagen chains are not known in detail no information is available on the primary structures of collagens of different species The amino acid compositions have however been studied by many investigators

Tables I II VI and VII present the relative proportions of individual amino acids and their ranges which show that regardless of the species all collagens are closely similar in amino acid composition The ratio of *polar* and *nonpolar* amino acids is constant the latter comprising about two thirds of all amino acids The invariable content of glycine one third of the total is a condition for the triple helical conformation The proportion of *acidic* and *basic* amino acids is rather constant although large variations occur in the proportions of lysine hydroxylysine and histidine The total number of lysine and hydroxylysine residues is constant except in hagfish collagen The great variation in histidine content may only be

apparent and due to the low proportion present The number of *hydroxyl groups* remains constant even though the proportions of serine, threonine and hydroxyproline vary greatly The significance of the variation of hydroxyl groups and *imino acids* will be discussed later The only *sulphur containing* amino acid in vertebrate collagens is methionine

Comparisons of amino acid compositions of collagens of different species show that amphibian fish and cyclostome collagens contain less imino acids than collagens of higher vertebrates Eastoe (1957) proposed that the low proportion of hydroxyproline in fish collagens is compensated for by a high proportion of aliphatic hydroxy amino acids Like the contents of serine and threonine the content of methionine decreases with evolution

Since the amino acid sequence of collagen is unknown phylogenetic comparisons of the primary structures of collagens of different species cannot be carried out as in the case of haemoglobin (Buettner Janusch and Hill 1965) cytochrome c (Margoliash 1963) and fibrinopeptides (Blomback Doolittle and Blomback 1965) Therefore only the amino acid compositions can be compared The sums of the differences between the relative numbers of amino acid residues in collagens of different species given in Table VI can be compared in Table XII To eliminate errors

Table XII

Differences between the amino acid compositions of citrate soluble collagens given in Table VI

	[illegible]	Chick	Frog	Flounder	Dogfish	Rayfish	Hagfish
Chick	30 5						
Frog	[illegible]	71					
Flounder	[illegible]	85	31				
Dogfish	[illegible]	[illegible]	[illegible]	6			
Rayfish	110	10	00	91	[illegible]		
Hagfish	10	11	[illegible]	36	[illegible]	85	
Lamprey	[illegible]	[illegible]	[illegible]	30	[illegible]	83	48

arising from possible differences in hydroxylation the proportions of imino acids and the proportions of lysines were taken instead of the proportions of the individual amino acids The figures in Table XII do not reveal two consecutive changes of primary structure that did not lead to an altered amino acid composition In general, however the differences in the amino acid compositions are related to the evolutionary ages of the species The amino acid composition of lamprey collagen is an exception and differs as has already been mentioned from the amino acid composition of hagfish collagen

The polar regions along the tropocollagen molecule can be located by means of the typical cross striated pattern of its segment long spacing collagen Great similarities are observed on comparing SLS collagens of the present investigation and the pattern of squid SLS collagen (Pikkarainen *et al* 1966 unpublished work by the author in Prof Kuhn s laboratory in 1967) Quite recently, Nordwig and Haydul (1967) did not find any differences between the SLS patterns of collagens of *Actinia equina* L a sea anemone and a calf

SECONDARY STRUCTURE

A triple helical conformation in crystalline regions is common to all collagens Regardless of the vertebrate species the helix content was constant in the citrate soluble collagens of the present investigation This was shown by the approximate constancy of the differences between the specific rotations of native and denatured collagens as well as the constancy of the quantities $A^{H} - A^{D}$ and $b_0^{H\ D}/a_0^{H\ D}$

The functional evolution of collagen is characterized by an increase in structural stability This is experimentally observed as a higher denaturation temperature of the collagen In the present study the stabilities of collagens to heat measured in both the solid and the liquid state show that the cold blooded animals clearly differ from warm blooded animals in having a weaker collagen structure A comparison of either the T_s or the T_D values (Tables IV and IX) reveals that although an evolutionary increase in the stability of collagen has occurred a large difference exists between various species of the same class e g between dogfish and rayfish and between hagfish and lamprey The material is too small to permit one to draw any conclusions about the natural selection of mutations in the evolution of collagen stability The apparent differences between the

apparent and due to the low proportion present The number of *hydroxyl groups* remains constant even though the proportions of serine threonine and hydroxyproline vary greatly The significance of the variation of hydroxyl groups and *imino acids* will be discussed later The only *sulphur containing* amino acid in vertebrate collagens is methionine

Comparisons of amino acid compositions of collagens of different species show that amphibian fish and cyclostome collagens contain less imino acids than collagens of higher vertebrates Eastoe (1957) proposed that the low proportion of hydroxyproline in fish collagens is compensated for by a high proportion of aliphatic hydroxy amino acids Like the contents of serine and threonine the content of methionine decreases with evolution

Since the amino acid sequence of collagen is unknown, phylogenetic comparisons of the primary structures of collagens of different species cannot be carried out as in the case of haemoglobin (Buettner Janusch and Hill 1965) cytochrome *c* (Margoliash 1963) and fibrinopeptides (Blomback, Doolittle and Blombäck 1965) Therefore only the amino acid compositions can be compared The sums of the differences between the relative numbers of amino acid residues in collagens of different species given in Table VI can be compared in Table XII To eliminate errors

Table XII

Differences between the amino acid compositions of citrate soluble collagens given in Table VI

	Pig	Chick	Frog	Flounder	Dogfish	Rayfish	Hagfish
Chick	30 5						
Frog	[illegible] 5	71					
Flounder	[illegible] 5	85	31				
Dogfish	63 5	57	58	[illegible]			
Rayfish	119	[illegible]	90	91	54		
Hagfish	[illegible] 5	113	51 5	36	85	85	
Lamprey	[illegible] 5	69	6	30	[illegible]	83	48

stereochemically identical whereas the hydroxyl group of hydroxyproline deviates slightly from these Replacement of serine by threonine or *vice versa* in the chain is thus not accompanied by a change in the hydrogen bond energy of the hydroxyl group as would be in the case if either were replaced by hydroxyproline

The way in which hydroxyl groups stabilize the collagen structure is unknown Combined O and N acetylation diminishes the T_S value of calf skin collagen from 64°—66° to 40°—44°, while N acetylation alone has no effect (Gustafson 1954b) If we accept the Rich Crick type II model of collagen structure, the hydrogen bond formed by hydroxyproline is intermolecular (intramolecular in type I) The investigations of Harrington (1964), Bensusan and Nielsen (1964) and Rao and Harrington (1966) support the Madras structure with two interchain hydrogen bonds to every amino acid triplet (only one hydrogen bond in the Rich Crick types I and II) One hydrogen bond per triplet is, however enough to produce the triple helical conformation as demonstrated by the studies of Rogulenkova Millionova and Andreeva (1964) and Traub and Yonath (1966) on the synthetic polypeptides poly (Gly Pro Hypro) and poly(Pro Gly Pro) Since the ordered structure is found in limited regions throughout the molecule it is not impossible that both structures those with one and two stabilizing hydrogen bonds to each amino acid triplet should be taken into consideration

The higher heat stability of the secondary structure of mammalian and avian collagens compared to that of lower vertebrate collagens leads to speculations about the connections between imino acid serine and threonine contents and high body temperatures of higher vertebrates and homoiothermy in general

TERTIARY STRUCTURE

The citrate soluble collagens examined in the present study were separated into three types by gel electrophoresis and CM cellulose chromatography In gel electrophoresis the separation is determined by both the charges and the sizes of the particles whereas the charge is the principal resolving property in CM cellulose chromatography

At constant ionic strength the electrophoretic mobility is directly proportional to the particle charge The thickness of the ionic double layer surrounding and the hydrodynamic volume taken up by the particle are

constant The experiments performed to determine the effect of a change in ionic strength gave gel electrophoretic patterns similar to those obtained when the pH was varied Jackson and Neuberger (1957) have shown that an increase in the ionic strength of a collagen solution leads to a decrease in the isoelectrophoretic point of the collagen This has been attributed mainly to increased binding of anions Another consequence of an increase in ionic strength is that the hydrodynamic volume is reduced and the ionic double layer moves in towards the particle and alters the net charge of the particle (Bull 1964)

Comparison of the behaviour of various α chains in Figs 9—25 reveals that the mobility of the $\alpha 2$ chain is highly dependent on ionic strength and pH (Figs 9—10), while the mobilities of the $\alpha 1$ and α chains (rayfish type) resemble each other in being independent of these two parameters The similarity of chains $\alpha 1$ and α was further confirmed in the experiments where the effect of gel concentration was studied (Figs 27 and 29) and in the gel electrophoresis of collagen mixtures (Fig 7) The influence of ionic strength or pH on migration is greater for the β component of the rayfish type collagen than for the β component of the guinea pig type

Investigations on the influence of gel concentration on electrophoretic mobility revealed that the α and β components (which differ in molecular weight) behaved differently when the ionic strength was changed at the same gel concentration (Figs 26—29) This can be explained by assuming that the decrease in ionic strength has a greater effect on the hydrodynamic volume of the particle than on its charge The effect of gel concentration on the migration of chains $\alpha 2$ and $\alpha 1$ of guinea pig and the chain α of rayfish collagen (which chains are of equal molecular weight) at ionic strength 0 020 resembled the effect of a decrease in either ionic strength or pH (Fig 25) Since starch is known to contain charged groups the explanation could be a change in the ionic strength resulting from the variation in gel concentration

Lamprey collagen clearly occupies a special position on the basis of its chain composition and chain behaviour when compared to other collagens This exceptional position is confirmed by amino acid analyses Another exception is flounder collagen Analyses of collagens of other flatfishes living in the same kind of environment might possibly shed more light on this finding

The appearance of the bony fishes seems to have been a turning point in the development of collagen perhaps because the collagen became more easily ossified On the basis of the present results the following hypothesis of the evolution of α chains is proposed (Fig 39) The primitive collagen

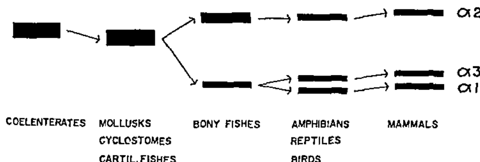


Fig 39

molecule was composed of three identical α chains Possibly as a result of two successive duplications of the collagen gene three α chains developed which underwent independent mutations leading to the three different α chains of the higher vertebrates (Pikkarainen and Kulonen 1967)

QUATERNARY STRUCTURE

The *collagen fibres in tissues are formed from tropocollagen molecules* which aggregate according to recent views side to side and overlap a quarter of their length This model is not however generally accepted (*e g* Grant Horne and Cox 1965) The periodicity of 640/700 Å observed in native collagen fibre in electron microscopy is related to the mode of aggregation The absence of periodicity in native fibres of *Lumbricus* and *Ascaris* collagens and of vitrosin is ascribed to the fact that the basic molecule is not tropocollagen but a polymer of this (Maser and Rice 1963a Beer Josse and Harrington 1964 and Olsen 1965)

The solubility of collagen reflects the stability of the quaternary structure Interspecies comparisons have shown (Table X) that up to ninety per cent of the native collagens in cyclostomes and fishes is soluble while the corresponding proportion for native collagens of full grown higher vertebrates is only a few per cent The increase in stability during evolution is reflected also in the case of gelatinization of the insoluble collagen (Table XI)

An increase in the intermolecular attractive forces perhaps an increase in the number of cross links could be the reason for the decreased solu

bility The histological differences laminated fibre structure *versus* three dimensional, cannot alone explain the differences in solubility Parallel fibres are found in mammalian achilles tendon without the solubility being high There may also exist differences between mammalian and the more primitive fish collagens in their ability to form stable aggregates

As shown in Table X a decrease in the solubility of collagen occurs in mammals with age It might thus be possible to study the process of aging by comparing animals that differ in evolutionary age if this age dependent change in collagen solubility could be taken as a recapitulation

The existence of the biphasic denaturation curve which was a constant finding in the determinations of shrinkage temperatures of ray fish, hagfish and lamprey skins could be explained by assuming the presence of two types of chemical bond that differ in stability In these animals the second shrinkage was abrupt in contrast to a very slow shrinkage of flounder and dogfish skins The two-stage shrinkage is in agreement with the two stage denaturation curve of collagen solutions (Engel 1961) Rigby (1961, 1967) was able to show that thermal shrinkage proceeded in two stages also in urea, dilute hydrochloric acid and dilute sodium hydroxide solutions and that the first temperature T_T coincided with T_D

CONCLUDING REMARKS

As a concluding remark the author would like to put forward the following hypothesis of the evolution of collagen The basic molecule tropo collagen is in the primitive form composed of three identical peptide chains in triple helical conformation This structure has developed into another that differs from the primitive structure by having all chains dissim ilar probably as a result of repeated duplications of the collagen gene followed by independent changes in the DNA corresponding to each chain Mutations involving single amino acid replacements have been permitted as long as they have not given rise to changes in the tertiary structure of collagen

An essential feature in the evolution of collagen seems to have been the partial replacement of serine and threonine by proline (because of the close similarity of their codons) which is converted to hydroxyproline The structure of collagen evidently presupposes a certain fairly constant con tent of hydroxyl groups Thus the contents of imino acids hydroxyproline serine and threonine all correlate with the stability of collagen, the last two contents however negatively

The lower solubility of collagens of higher animals at both low and high temperatures is due to more close packed arrangements of tropocollagen molecules and represents development occurring at the level of the quaternary structure of collagen This same phenomenon is observed also in the development of an individual

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Last but not least I wish to thank my wife for her sympathetic tolerance of my continual absence from the family circle

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 311

INACTIVATION OF 5-HYDROXY-TRYPTAMINE IN RATS

WITH SPECIAL REFERENCE TO THE ROLE OF THE LIVER

BY

ERKKI S HEIKKINEN

OULU 1968

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 311

FROM THE DEPARTMENT OF SURGERY UNIVERSITY OF OULU AND
THE DEPARTMENT OF PHARMACOLOGY UNIVERSITY OF OULU

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PREFACE

The subject of the present study was suggested to me by Professor Teuvo Larmi, M D Head of the Department of Surgery, University of Oulu He supported, encouraged and inspired me in all phases of the work I wish to express my gratitude to him

The investigation was carried out in the Institute of Pharmacology, University of Oulu, and my respectful thanks are due to the Head of the Institute Professor Niilo Karki M D, for placing the facilities of his Institute at my disposal and an expert on serotonin offering constructive criticism valuable advice and guidance during the work

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Oulu November 1967
Erkki Heikkinen

CONTENTS

I INTRODUCTION AND REVIEW OF THE LITERATURE

1 History and distribution

Some twenty years ago scientists were occupied with the following two unsolved problems Some sought an explanation for the vaso constrictive property of defibrinated blood not present in the plasma Others studied the endocrine character of the intestinal enterochromaffin cell system The riddle of the vasoconstrictive property of the serum was solved in 1948 when serotonin was successfully isolated from bovine serum (Rapport et al 1948) Its chemical structure was recognized as 5 hydroxytryptamine (5 HT) (Rapport 1949) Since the 1930 s pharmacological studies had been carried out by Erspamer (1940) with enteramine the hormone of the intestinal enterochromaffin or argentaffin cells with a contracting action on the smooth muscles But only after the chemical synthesis of 5 HT had been successful (Hamlin and Fisher 1951 Speeter et al 1951) could Erspamer and Asero (1952) identify by paper chromatography, the enteramine as 5 hydroxytryptamine creatinine sulphate

5 HT therefore helped to solve the two problems which had been believed to be entirely separate Subsequently 5 HT itself has become the subject of extremely intense research A minimum of 2 500 papers on this amine have been published in about 15 years On the basis of separate studies general summaries have been published on its incidence biosynthesis metabolism and its physiological and pharma cological effect (Page 1954 Erspamer 1954, 1955 1961, 1966, Cerletti 1958, Lewis 1958, Lembeck 1961 Robson and Stacey 1962 Varley and Gowenlock 1963 Garattini and Valzelli 1965 Paasonen 1965)

Despite the immense scientific interest in 5 HT the part played by this biogenic amine in the human organism is still unclear Ninety per cent of the 5 HT of the human organism is situated in the gastrointestinal mucosa (Sjoerdsma 1959) The blood is its second biggest storage place (Erspamer 1955, 1961 Jepson 1963) Consequently, the principal localizations are in the same tissues in which the pharmacological effects of 5 HT aroused attention long ago On the basis of this distribution in the organism 5 HT has been assumed to participate in one way or another in the control of the functions of the alimentary canal and the organs of blood circulation (Erspamer 1961) Of the many potential physiological functions of 5 HT, its participation in the regulation of intestinal motility is perhaps most conclusively shown (Bulbring and Lin 1958, Bulbring and Crema 1959 a b Peart and Robertson 1961)

2 *Gastrointestinal biosynthesis, storage and release*

The mammalian organism synthetizes 5 HT from the L tryptophan it receives in food (Udenfriend et al 1953) A human being normally utilizes only 1 per cent of the dietary tryptophan for 5 HT production while the bulk is required for the production of protein and nicotinic acid (Sjoerdsma et al 1956)

The biosynthesis of 5-HT is considered diphasic The amino acid is first transformed by means of tryptophan 5 hydroxylase into 5-hydroxytryptophan (5-HTP) and again, by catalysis of decarboxylase, into 5 HT (Sjoerdsma et al 1955a) There are several opinions on the location of this synthesis performed by the organism and its different phases 5 HTP decarboxylase activity is noted in several tissues for example kidneys intestine, liver and brain (Gaddum and Giarman 1956 Tissari 1966) whereas remarkable tryptophan 5 hydroxylase activity has been shown only in the intestinal mucosa and to some extent in the renal tissue (Cooper and Melcer 1961) A fairly common assumption is that at least some of the 5 HTP produced by the argentaffin cells passes unchanged into other tissues in which it rapidly turns into 5 HT (Dalgliesh and Dutton 1957) In this way for example the brain would receive serotonin which, being lipid insoluble poorly crosses the blood brain barrier (Erspamer 1961) 5 HTP on the other hand passes easily through this barrier (Udenfriend et al 1957 a)

Although using relatively high substrate concentrations, 5 hydroxy-

indoles have been produced from tryptophan on liver extracts (Freedland et al 1961 Ross and Haljasmaa 1966), it seems improbable that the hydroxylase of the liver played a part in the physiological 5 HT synthesis of the organism (Renson et al 1962)

Abundant evidence has accumulated in favour of the general idea that the argentaffin Kultschitzky (1897) cells of the intestine synthesize store and liberate 5 HT

Resection of any degree of the gastrointestinal canal of the rat reduces the 5 HT contents of the other tissues and the secretion of its metabolite the 5 hydroxyindole 3-acetic acid (5-HIAA) (Bertaccini 1960) Human subjects in whom considerable parts of the alimentary canal have been removed, secrete subnormal quantities of 5-HIAA into the urine (Haverback and Davidson 1958 Rosenberg et al 1959 Bertaccini and Chieppa 1960)

Large quantities of 5 HT are stored in the argentaffin or chromaffin cell granules in the gastrointestinal mucosa (Vialli and Erspamer 1933, Erspamer 1954 1955) A distinct correlation exists between the amount of argentaffin cells and the 5-HT stores (Erspamer 1961) The wellknown liberator of 5 HT reserpine (Brodie et al 1957), abolishes the argentaffinity of the cells (Benditt and Wong 1957, Penttila 1967) Histochemical studies have shown that the cytoplasm granule fluorescent in formalin fixation and with argentaffin or chromaffin reaction contain 5 HT and that the specific histochemical properties of the granules are due to this amine (Barter and Pearse 1953 1955 Benditt and Wong 1957, Campbell 1963) According to estimates the human argentaffin cells contain 5000—11000 µg 5 HT per gram (Gowenlock and Platt 1963 Penttila 1967)

Measurable quantities of 5 HT are liberated from the intestinal stores on stimulation of intestinal motility (Bojs 1961) or on increase of its intraluminal pressure (Bulbring and Crema 1959a Karki et al 1960 Warner et al 1966 a) on acidification of the intestinal contents (Resnich and Gray 1962) and on intraluminal administration of hypertonic glucose (O Hara et al 1959, Peskin and Miller 1962, Drabanas et al 1962 Sloop et al 1962 Walker et al 1962) As suggested by these experimental studies hypermetabolism of 5 HT has been noted in clinical material with the dumping syndrome (Howe 1964 Smith and Zeitlin 1966) and with mechanical obstruction of the small intestine (Warner et al 1966 b) Admittedly some studies associated with these diagnoses have led to results incompatible with those indicated above (Silver et al 1965)

Apparently the gastrointestinal stores liberate 5 HT continuously into the circulation, for the blood of the portal vein has been found to contain more 5-HT than that of the hepatic vein (Johnson et al 1961 Drabanas and McDonald 1963) or the peripheral arterial blood (Toh 1954)

The carcinoid tumour (Oberndorfer 1907) is probably the best evidence that argentaffin cells can synthesize, store and liberate 5 HT The classical carcinoid, by its pathological anatomy is an argentaffinoma (Masson 1928), it is located in those parts of the gastrointestinal canal developing from the midgut of the embryo in other words, the jejunum, ileum, caecum or proximal colon (Williams and Sandler 1963) This carcinoid is the most common tumour of the small intestine in surgical material it is responsible for 20—30 per cent of the malignant tumours of this intestine (Franssila 1966) Carcinoids of atypical histochemical qualities and biological activity are seen primarily in the bronchus the stomach, biliary and pancreatic ducts, and in the distal colon (Sandler et al 1961, Gowenlock and Platt 1963 Williams and Sandler 1963) The carcinoids of the distal colon are biologically inactive and their cells are neither argentaffin nor chromaffin (Williams and Sandler 1963) On the other hand the typical argentaffinoma contains 5 HT (Lembeck 1953) Even a small tumour can produce an observable increase in 5 HIAA secretion suggestive of excess production of 5 HT (Snow 1963)

The carcinoid syndrome (Bjork et al 1952) usually develops only after the intestinal tumour has sent metastases into the liver or when the primary tumour is located outside the splanchnic region (Thorson 1958 Hedinger 1958 Waldenstrom 1961) Essential parts of this syndrome are hyperserotoninaemia (Pernow and Waldenstrom 1954) and profuse 5 HIAA secretion (Sjoerdsma et al 1955b) Apart from the mastocytoma of mice the carcinoid is the only tumour konwn to provoke excess production of 5 HT (Erspamer 1961)

For the reasons given above the general opinion (Erspamer 1961) seems well founded which says that the extracerebral 5 HT or 5 HTP in the mammalian organism, excepting the mast cells of mice and rats (Levine et al 1964a), derives from the argentaffin cells of the gastrointestinal canal

3 Inactivation

The organism is well equipped for the inactivation of 5 HT Most of the 5 HT injected into man or animal is secreted during the first 8—24 hours in the form of various metabolites mainly into the urine (Udenfriend and Weissbach 1958, Donaldson and Gray 1959, Keglevic et al 1959, Karki and Paasonen 1959, McIsaac and Page 1959 Peltola and Leppanen 1960, Bojs 1961, Erspamer and Bertaccini 1962 Airaksinen 1963)

5 HT is a potent biogenic substance Normally most of 5 HT is inactive »bound» to the intracellular space, while extremely small quantities occur in a free active form susceptible to metabolism (Cerletti 1958 Erspamer 1961, 1966 Garattini and Valzelli 1965, Paasonen 1965)

The binding or diffusion of free 5 HT into the tissues may be considered a biological mechanism of inactivation (Blaschko 1958 Udenfriend 1958) Thrombocytes are inclined to absorb 5 HT rapidly from the plasma against a considerable concentration gradient (Humphrey and Toh 1954 Hardisty and Stacey 1955) In normal conditions the platelets are far from saturated with 5 HT, and they are able to double or even treble their 5 HT content from a low environmental amine concentration (Weissbach et al 1958a Born and Gillson 1959 Hughes and Brodie 1959 Stacey 1961) The opinions as to whether 5 HT occurs freely in the plasma and on the stability and liberation mechanism of 5 HT bound to thrombocytes are contradictory (Udenfriend and Weissbach 1958 Born and Gillson 1959 Hughes and Brodie 1959 Bojs 1961 Crawford 1966)

The storage of 5 HT in the tissues is of course only a temporary solution in the inactivation of free endogenous or exogenous 5 HT The passage of 5 HT from the blood into most tissues is restricted since this amine ionized and insoluble in lipids poorly crosses the histo haematic barrier (Erspamer 1961, Bulat and Supek 1967)

Enzymatic oxydative deamination is the most important method of the organism for degradation and inactivation of 5 HT Monoamino oxydase (MAO) catalyses the conversion of 5 HT into 5 hydroxyindole-acetaldehyde (Blaschko 1952 Titus and Udenfriend 1954 Sjoerdsma et al 1955a Udenfriend et al 1956) Under the action of aldehyde de hydrogenase this 5 hydroxyindole acetaldehyde turns into 5 HIAA which is secreted into urine either as it is or as phenol conjugates (McIsaac and Page 1959 Airaksinen 1963) Several organs such as liver

lungs, brain, and intestine, contain MAO and aldehyde dehydrogenase (Levine and Sjoerdsma 1962, Klingman 1966, Tissari 1966) Many tissues are capable of metabolizing 5 HT, even the blood to some extent (Paasonen 1961 Pletscher et al 1966)

5 HIAA is not the only metabolite of 5-HT (Dalgliesh and Dutton 1957, Weissbach et al 1958b, Keglevic et al 1959 Chadwick and Wilkinson 1960 Airaksinen 1963) The inhibition of MAO does not prevent a rapid metabolism of 5 HT (Udenfriend et al 1957 b) N and O conjugates of 5 HT have been isolated from the urine of carcinoid patients (Airaksinen 1963) The O conjugates of 5 HT are biologically almost inactive whereas some N conjugates are highly potent agents (Axelrod 1961, Erspamer 1961) On intravenous administration of large doses of 5 HT to rats, the 5-HIAA, 5 HIAA O sulphate, 5 HIAA O glucuronide 5 HT O glucuronide and 5-HT O sulphate constitute about 97—98 per cent of the 5 HT metabolites secreted into the urine (Airaksinen 1963) Since these metabolites are inactive, the metabolism of 5 HT is generally likely to result in biological inactivation

A small proportion of the 5 hydroxyindole acetaldehyde is reduced to the corresponding alcohol, 5 hydroxytryptophol a fairly active compound (Kveder et al 1962 Paasonen and Airaksinen 1965, Pletscher et al 1966) Ingestion of ethyl alcohol highly significantly raises the proportion of 5 hydroxytryptophol among the 5 HT metabolites (Davis et al 1966)

Small amounts of free 5 HT leave the organism in free form in urine and feces (Keglevic et al 1959, Airaksinen 1963) It is assumed that the ceruloplasmin of the plasma also takes some part in the degradation of 5 HT (Porter et al 1957, O Reilly and Loncin 1967) with resulting p quinone-imine derivatives Apparently the organism is also capable of rupturing the indole ring since even volatile compounds of labelled 5 HT may be formed (Keglevic et al 1959, Erspamer 1961)

The liver apparently plays an important part in the degradation of 5 HT It has the highest MAO and aldehyde dehydrogenase activity of the organism (Klingman 1966) Furthermore the liver can use the mechanisms of glucuronide and sulphate conjugation and of acetylation and methylation, for detoxication of substances (Seligson 1963) The liver homogenate decomposes 5 HT effectively producing 5-HIAA and conjugates of 5 HIAA and of 5 HT (Titus and Udenfriend 1954 Udenfriend et al 1956, Chadwick and Wilkinson 1960

It is also known that on perfusion of isolated liver, 5 HT disappears rapidly from the prefusate (Levine et al 1964b Moore and Eiseman

1966) These experimental studies did not show however, whether the liver in vivo is able to control the access into systemic circulation of 5 HT liberated from the argentaffin cells as has been assumed to occur in carcinoid (Thorson 1958, Waldenstrom 1961, Gowenlock and Platt 1963, Snow 1963, Hallen 1964)

In addition to perfusion of organs in vitro two in vivo investigations have been performed with dogs whose hepatic circulation was loaded with an endoportal infusion of 5 HT (Johnson et al 1961 Drabanas and McDonald 1963) The results were contradictory In Johnson s experiments increasing the 5 HT content of the portal blood up to 500 per cent increased almost accordingly the content in extraportal circulation The result reported by Drabanas was that the intact dog liver could at its best eliminate 80 per cent of the 5 HT infused into the portal vein during a period of infusion of 15—20 minutes

Besides liver the isolated and intact lung has also been found to remove 5 HT from the blood (Gaddum et al 1953 Eiseman et al 1964, Crawford 1965 Davis and Wang 1965)

4 5 HT and haemodynamics

5 HT is a vasoconstrictor (Rapport et al 1948, Page 1958) Notwithstanding this intravenously injected 5 HT often increases the blood flow of peripheral organs with the exception of local vascular spasm (Sjoerdsma 1959), reducing peripheral resistance (LeMessurier et al 1959, Fox 1961) and raising the cardiac output (Reid 1952 Page 1958 Bojs 1961)

The effect of pharmacological doses of 5 HT on blood pressure is a complex issue difficult to explain The response has been found to depend on the animal species (Page and McCubbin 1953 Schneider and Yonkman 1954) on the amount of the dose and the existing blood pressure (Outschoorn and Jacob 1960), on neurogenic vasotonia (Page and McCubbin 1953) and on the site of administration (Erspamer 1961) An intravenous 5 HT injection has very often produced either a biphasic or triphasic blood pressure response with both hypotensive and hypertensive phases (Reid 1952 Page and McCubbin 1953 Salmoiraghi et al 1956) 5 HT has usually produced an exclusively tachycardiac effect on the pulse rate (Page 1958 Bojs 1961)

Although excess production and accelerated metabolism of 5 HT are diagnostic to the carcinoid syndrome, the increase in the amount of 5 HT has not sufficed to account for all the haemodynamic symptoms associated with the disease (Peart and Robertson 1961, Melmon et al 1965)

The hemodynamic flush of the human skin can sometimes be provoked by an exogenous 5-HT injection and infusion (Roddie et al 1955 Bojs 1961), while exogenous 5 HT frequently produces no observable vasodilatation in the skin (Fox et al 1961 Oates et al 1964, 1966) Efforts have therefore been made to explain the pathogenesis of this symptom from histamine production or liberation associated with the syndrome (Feldberg and Smith 1953, Pernow and Waldenstrom 1957, Moore et al 1963 Oates and Melmon 1966) It has also been suspected that the atypical flush often seen in association with bronchial and gastric carcinoids, is produced by 5 HTP (Sandler and Snow 1958 Sandler et al 1961) High plasma kinins have been recorded in patients with carcinoid and dumping syndromes during the attacks (Oates et al 1964, Zeitlin and Smith 1966) It has been assumed that the kallikrein the tumour may secrete liberates bradykinin from the plasma which then causes vasodilatation in the skin (Oates et al 1966, Oates and Melmon 1966)

II OBJECT OF THE PRESENT STUDY

Since the carcinoid syndrome is seen only when tumour tissue secreting 5 HT occurs in the area of extraportal circulation, it is apparent that the liver if required is able effectively to control the passage of 5 HT into extraportal circulation Not enough evidence has been brought together, by the experimental studies to date, for hepatic action of this kind The two investigations performed into the degradation of endoportally administered 5 HT in the intact liver of dog led to contradictory results (Johnson et al 1961, Drabanas and McDonald 1963)

The organism, if necessary, probably takes recourse to several simultaneous mechanisms of 5 HT inactivation such as the absorption capacity of the thrombocytes and the enzymatic forces of the different organs How important a part the liver represents in the 5 HT inactivation capacity of the whole organism, is not known

The pharmacological effects of extraportally administered 5 HT on haemodynamics have been much studied and many different explanations have been offered On the other hand, the cardiovascular responses of exogenous 5 HT infused into the portal vein have not been analysed, yet this route of administration apparently imitates the passage of endogenous 5 HT from the areas of synthesis into the circulation much better than a 5 HT injection straight into the extraportal veins

The object of the present study was to develop *a serviceable method of perfusion for intact rat liver* and to utilize it to study the following three questions

(1) How effective is the ability of the liver to control the passage of 5 HT, infused by the portal route into extraportal circulation?

(2) To what extent and when does the organism have to resort also to the extrahepatic mechanisms of 5 HT inactivation when 5 HT is infused endoportally?

(3) Is there any correlation between the amount of 5 HT entering the extraportal circulation and the haemodynamic effects observed?

SCHEME OF THE METHOD

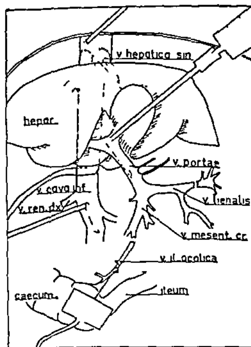


Fig 2 Schematic drawing of the method of perfusion Aspiration needles in the portal vein hepatic vein and the inferior vena cava Infusion needle in the ileo colic vein In addition the linen thread around the inferior vena cava is visible

the needles were in position the retractors were removed and the wound was covered with a warm compress A thermometer was inserted in the rectum and the lamp above the operating table was used when required to keep the animal warm so that the rectal temperature remained at 36—38°C

To record the pulse rate and ECG the needle electrodes of »Physio graph Six» were inserted on both sides of the thoracic cage The prevailing portal pressure could be read from the height of the fluid column in the catheter of the aspiration needle or was recorded by the Physiograph (Fig 3)

Perfusion

5 HT (serotonin creatinine sulphate Monohydrat[R], Fluka AG Buchs SG) was infused at a constant rate 0 2 ml/min , in iso onic physiological saline into the mesenterial vein The 5 HT contents of the solutions varied 25 μg/ml 50 μg/ml, 75 μg/ml 100 μg/ml, 400 μg/ml

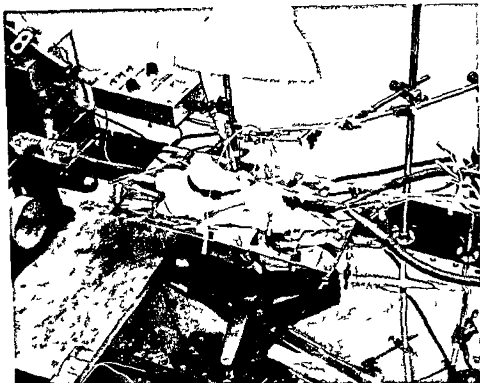


Fig 3 General view of the method of perfusion employed Th anaesthetized rat is fixed to the operation table the trachea and left carotid artery are cannulated the rat s opened abdomen is covered with compress soaked in physiological saline the aspiration needles are seen fixed in position as is the infusion needle connected to the continuous infusion equipment In addition a needle electrode is seen in the left thoracic cage and thermometer in the rectum

600 µg/ml 800 µg/ml and 1600 µg/ml Perfusion usually continued fo 30—40 minutes, though in the early experiments for only 10—15 minutes

Since the quantity of blood in the experimental animals was small samples were not aspirated simultaneously from the different points but successively and efforts were made to alternate the order in which samples were drawn The exact time to the nearest minute, of aspiration was recorded The samples were drawn into 1 ml plastic syringes The anticoagulant was 0 1 ml of 1 per cent EDTA (Disodium Ethylene Diamine Tetra Acetate) The volume of each sample was 0 6 ml In addition one sample with 1 2 ml volume was drawn from each of 95 rats half of this was used as an internal standard sample (see p 27)

To secure plasma without decomposing the thrombocytes, the blood was not aspirated into syringes but dripped along siliconized needles and plastic catheters straight into plastic centrifuge tube The hepatic vein sample was taken by introducing a plastic cannula to the hepatic vein from a superficial jugular vein

Discussion

General anaesthesia and laparotomy are a stress on the organism There is no doubt that they affect the metabolism e g tending to reduce the biotransformation of substances in the liver (Van der Decken and Hultin 1960) The small doses of barbiturate used and the light ether anaesthesia do not apparently in themselve markedly slow down the detoxication processes of the liver, since even triple doses of barbiturate combined with ether did not reduce the eliminat on of bromsulphalein in rabbits or humans (Bourne et al 1930)

Anaesthetics and 5-HT usually potentiate one another s action (Correll et al 1952) Tracheostomy and artificial respiration however, according to studies on rats, remove almost all the toxicity increase of 5 HT in connection with anaesthesia (Tammisto 1965)

The liver cells are particularly sensitive to hypoxia (Sherlock 1963) O was not measured but the pCO pH standard bicarbonate and base excess (Astrup) of the venous blood were determined in connection with three control perfusions It was found that the carbon dioxide pressure ranged from 40 to 25 mmHg and tended to fall slightly during the experiment The constant respiration used may therefore be considered adequate The fall in carbon dioxide pressure was associated with a slight fall in standard bicarbonate The pH of the blood remained unchanged during the experiment and varied in different animals from 7 30 to 7 35 The perfusion method, therefore, did not produce in the organism any such changes of the acid base balance as would have essentially modified the enzymatic function

Since the blood removed as samples was replaced with infusion of physiological saline the result was haemodilution and normocytic anaemia The microhaematocrit of six experimental animals was observed and showed a fall of 14 per cent on an average from the control sample to the last sample Rats weighing 250—300 g have about 20 ml of blood (Carthland 1928 Ormond and Rivera Velez 1965) A maximum of one fi[illegible] of this [illegible] was aspirated during the experiments (seven

samples) Normovolaemia and maintenance of adequate circulation are suggested by the fact that while physiological saline or mild serotonin solutions were infused, the pulse rate ECG, blood pressure and portal pressure showed no essential changes (Fig 20)

Rapid aspirations of blood samples were associated with a temporary fall in blood pressure whereas the portal vein pressure did not always change Friedman et al (1953) removed 0 7 ml blood per 100 cm of body surface from rats and found no changes in the caliber or blood flow of the intrahepatic vessels The volume drawn at one time in the present experiments was 0 5 ml that is to say, only about 0 3 ml/100 cm of body surface Hence the relative stability of the portal pressure complies well with Friedman s findings Provided no extravasation of blood occurred during the experiment both the arterial (>60 mm Hg) and the portal (>10 cm H O) pressures remained satisfactory throughout The experiment was considered a failure if the blood pressure owing to extravasation fell permanently below 60 mmHg before the last sample was drawn In this way efforts were made to eliminate from the series the animals in which hypoxia due to poor ti sue perfusion perhaps considerably modified the metabolism

If the needles were adequately introduced into the vessels 0 5 ml blood was easily drawn in about 10 seconds In these vessels the blood flow is profuse the rat s cardiac output being some 30 ml/min and portal flow some 10—15 ml/min (Takàcs et al 1959 Levine et al 1964 b)

The hepatic artery of the rat can be ligated without producing changes in the physiology or histology of its liver (Wiles et al 1952) Judging by this the share of the rat s hepatic artery in total hepatic circulation appears to be below 10—15 per cent a percentage obtained in dog experiments (Blalock and Mason 1936) the dog s hepatic artery is considerably more important for the liver than the rat s In the present study the hepatic artery was not ligated The dilution effect it may produce in endoportal amine infusions appears to be practically negligible

The circulation of the rat is very fast The transhepatic circulation time is only 2 3 seconds (Daniel and Prichard 1951) Any agent administered endoportally therefore spreads in a few seconds throughout the circulatory region From samples drawn also from the inferior vena cava, information could be obtained concerning the fate of the amine during extraportal circulation

In addition to the ventilatory and circulatory factors and those

associated with anaesthesia and operation the animal's age sex, food fasting and temperature may affect the biotransformation of substances in the liver (Dixon et al 1960, Sotaniemi 1967) Therefore the rats used were of the same age and same sex, fed on a standard diet and not kept fasting, their temperatures were checked during the experiments

B DETERMINATION OF 5 HT FROM THE RAT S BLOOD

The literature recognizes a large number of different methods of extracting and measuring 5 HT (Sandler 1963 Erspamer 1966) The number of the methods reported apparently suggests that the ideal method has not been developed Imperfection non-selectivity and destruction are difficult to avoid when 5 HT is extracted from the tissues The biological and chemical methods of measurement are not completely specific to 5 HT

Many authors give preference, among the chemical methods to the modification developed by Waalkes in 1959 (Sandler 1963 Garattini and Valzelli 1965) Despite its two alternative relatively complicated extraction procedures, it has been used in many experimental studies (Bojs 1961) 5 HT determinations on the whole blood of at least dogs (Peskin and Miller 1962, Drabanas and McDonald 1963) and rabbits (Moore and Eiseman 1966) have been made by this means Prior to the present study as far as is known it has not been applied to determination of the 5-HT levels of rat blood

Method of determination

It was necessary to modify the method of determining the 5 HT in the blood described by Waalkes (1959) to meet the requirements of the present study Since the rat s blood samples were only one fifth of the quantities of human blood Waalkes had used the solution volumes Waalkes had indicated for the precipitation of proteins (10 per cent $ZnSO_4 7H_2O$ and 1N NaOH) and to bind heavy metals (Saturated EDTA*) had to be reduced Experiments with other

*) Disodium Ethylene Diamine Tetra acetate

The t values were used to determine whether the serotonin difference noted between matched pairs differed statistically from zero

$p < 0.05$ = almost significant
$p < 0.01$ = significant
$p < 0.001$ = highly significant

The error of the method was given the form of standard deviation (SD) between the 5 HT values of identical blood specimens and expressed in the form of variation coefficient (V)

$$V = \frac{SD}{\bar{x}} \cdot 100$$

IV RESULTS

A RELIABLITY OF 5 HT DETERMINATION

Recovery of 5 HT from specimens of different types and the 5-HT contents of these specimens Influence of 5-HIAA on blood analyses Since the Waalkes (1959) method of determination had to be modified for the analyses of rat blood the reliability of the 5 HT concentrations obtained in different types of specimens was studied In addition it was useful to find out the extent to which the experimental conditions (anaesthesia laparotomy artificial respiration, etc) modified the 5 HT levels of rat blood

The endogenous 5 HT content of blood drawn under experimental conditions from the hepatic vein was determined for 95 rats by including in each determination procedure in addition to the blood specimens one blank specimen (water) one standard (water + 1 μg 5 HT) and one internal standard (blood + 1 μg 5-HT) specimen

Similarly, the 5-HT contents of blood taken from six rats by decapitation were determined

Five animals were given 1 5 mg reserpine (Reserpin[R] Laake Oy) intraperitoneally eight hours before blood was drawn under anaesthesia

The 5 HT levels of the plasma were determined for 11 animals under experimental conditions from control blood specimens drawn before 5 HT infusion

The blood 5 HT of 11 human subjects not known to suffer from any disease or to take any drugs affecting the 5 HT content of the tissues was measured

Table 1 The recovery of 5 HT from different types of samples Amounts of endo₆enous 5 HT in these samples

Sampl	N mbe of case	Add o of 5—HT μg	V l me of sample ml	R very of dd d 5—HT f om d ffe en sampl μg/ml M n ± EM	R o n pe	M on en of dog ou 5—HT in sampl μg ml ± SEM)	M an body we ght g ± SEM
Whole blood of rat taken under experimental conditions	95	1	0 50	0 82±0 01	82	1 09±0 03	282±3.2
Whole blood of rat taken on decapitation	6	1	0 50	0 81±0 03	81	1 08±0 06	247±10 4
Blood from reserpinized rats	5	1	0 50	0 71±0 05	71	0 11±0 01	242±28 3
Rat plasma taken under experimental conditions	11	1	0 50	0 97±0 04	97	0 04±0 01	279±10 4
Human venous blood	11	1	2 00	0 69±0 04	69	0 12±0 02	

*) Calculated on the basis of theoretical readings obtained by adding together the individual readings for sample and the serotonin standard
*) Corrected to allow for recovery

Table 1 shows the recovery of 5-HT from these specimens of different types and the endogenous 5 HT contents of the specimens

On analysis of internal standard samples of varying 5 HT concentration the spectrophotofluorometer gave readings which showed that the 5 HT content of the blood and the corresponding fluorescence were in direct proportion up to a concentration of 50 μg/ml When the 5-HT concentration was increased above 50 μg/ml intensity of fluorescence began to increase more slowly until finally it began to fall

The error of the method was studied by adding 1 μg 5 HT to ten blood samples drawn from the same rat with one aspiration The coefficient of variation so obtained was 0 007 μg/0 5 ml equalling 0 014 μg/ml

The error produced by 5 HIAA in the determination was the following

	Relative fluorescence
Sample	17
Sample + 1 μg 5 HT	40
Sample + 10 μg 5 HIAA	19
Sample + 100 μg 5 HIAA	28
Sample + 1 mg 5 HIAA	149
Blank	1

An addition of 1 μg 5 HT to the blood sample raised the fluorescence reading twice as much as 100 μg 5 HIAA did in other words 200 μg 5 HIAA in the samples equalled roughly 1 μg 5 HT

The 5 HIAA content of several plasma samples drawn from the hepatic vein was examined by thin layer chromatography (Stahl 1962) It appeared that plasma drawn from the hepatic vein during endoportal 5 HT infusion (80 μg/min) could contain up to 10 μg 5 HIAA per ml

When the animals prior to 5 HT infusion were given pheniprazine, an MAO inhibitor (JB 516) the plasma samples drawn during infusion no longer exhibited the 5-HIAA spot (Fig 5)

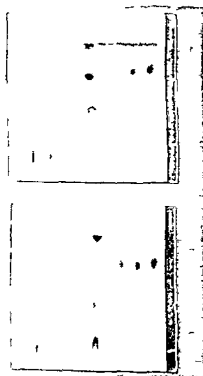


Fig 5 Thin layer chromatography 5 HT at a rate of 80 μg/min was infused into rats The top photograph shows in the middle an acetone extract (9 1) of 1 ml of hepatic vein plasma drawn during the infusion experiment from a rat not treated with MAO inhibitor (JB 516 pheniprazine) prior to the experiment, while the other photograph shows also in the middle a plasma extract from a rat to which 5 mg MAO inhibitor was given intravenously prior to the experiment Left of the central spot are the 5 HT standards (water + 0 5 μg water + 1 μg water + 2 μg 5 HT) and right the 5 HIAA standards (water + 0 5 μg water + 1 μg water + 2 μg 5 HIAA)

Discussion

The recovery of 5 HT from blood samples equalled satisfactorily that obtained by Waalkes (1959) with the butanol extraction method The extraction of 5 HT from the plasma was almost as complete as from the distilled water

The mean 5 HT content of the human blood, seen in Table 1, is exactly equal to the values quoted in the literature and obtained by both biological and chemical methods (Waalkes 1959, Schmid et al 1960, Ascroft et al 1964, Contractor 1964)

The 5 HT content of the rats whole blood, on the other hand, was considerably higher in the present than in most of the former studies (Erspamer 1961) But the 5 HT contents of rat serum indicated in the literature may be considered to comply well with the 5 HT contents of the whole blood now obtained Serum 5 HT concentrations up to 1 67 µg/ml have been recorded (Collier 1958) Bertaccini (1958) found that the 5 HT content of rat serum increased with increasing body weight He quoted a serum 5 HT concentration of 1 16 µg/ml for rats of the body weight used in the present study Since serum 5 HT probably equals only half the corresponding whole blood 5 HT (Hardisty and Stacey 1955) the whole blood 5 HT levels now obtained concur with those given by Bertaccini in his serum studies

The fact that the 5 HT concentrations in the whole blood of rats obtained in the present experiments were high could be due to several factors (1) Waalkes s sensitive chemical method has not been used earlier for 5-HT determinations in rats (2) The rats were large (3) The animals had free access to food and drink before samples were drawn (Sullivan 1960) (4) The samples were deepfrozen immediately, which reduced the loss of 5-HT during storage to a minimum (Garattini and Valzelli 1965 Tissari 1966) (5) The lysis of the cells was effective since the procedure included both freezing and mixture of the sample into a large volume of distilled water

The anaesthetics empoyed general anaesthesia and laparotomy dib not seem to affect the 5 HT contents of whole blood for no appreciable difference was demonstrable in 5 HT contents between blood taken by decapitation and that drawn in experimental conditions

Reserpinization on the other hand, reduced the 5-HT content of whole blood in eight hours to one tenth This 90 per cent reduction complies well with the results of the numerous studies concerning

the influence of Rauwolfia alkaloids on the tissue levels of 5 HT (Brodie 1958, Paasonen 1965)

Toh (see Garattini and Valzelli 1965) by biological determination has obtained a 5-HT content of 0 04 µg/ml for rat plasma Although the present method was not sensitive enough for the determination of 5 HT from plasma the result obtained 0 04 µg/ ml does not differ from Toh s result

Under the physiological conditions in which the amount of other 5 hydroxyindoles in the blood does not interfere the present chemical methods are highly specific (Waalkes 1959 Ascroft 1964, Contractor 1964) The selectivity of the method may however, be disturbed when the organism is being loaded with large amounts of endogenous or exogenous serotonin with a resulting increase in 5-HT metabolites

According to Airaksinen (1963) the 5 HIAA and 5 HT with their O conjugates constitute 97—98 per cent of the 5 HT metabolites secreted into rat urine if the 5 HT is injected intravenously at a high rate Since the fluorescence of the 5 HIAA O and 5 HT-O conjugates differs essentially in concentrated acid from the optimum fluorescence wave length of the corresponding non conjugated compounds (Airaksinen 1963) the free 5 HIAA apparently remains the only potential source of error when the 5 HT contents of blood and plasma are determined for animals treated with pharmacological doses of this amine The N conjugates of 5 HT are known to have and 5 hydroxy-tryptophol (free OH) apparently also has the same fluorescence spectrum as 5 HT (Sandler 1963) From the results of the quoted study by Airak sinen their proportion in the blood during 5 HT loading is apparently relatively low

The 5 HIAA contents of the plasma have not been studied during experimental 5-HT loading According to the present observations based on chromatographic studies up to 10 µg/ml of this rapidly excreted metabolite may have accumulated in the plasma during endoportal 5 HT infusion while the 5 HT levels of whole blood in systemic circulation did not yet exceed 2—3 µg/ml On the other hand 5 HIAA passed poorly through the extraction procedure 10 µg 5 HIAA increased the fluorescence of the blood sample only as much as did 0 05 µg 5 HT This result concurred with that reported by Quay (1963) in his studies of the extraction of the various indoles According to him the recovery of 5 HIAA and of N acetyl serotonin on extraction with alkaline salt impregnated n butanol was only 0—1 per cent while the recovery of serotonin was 92 per cent

On the basis of the above the error produced by the 5 HT metabolites formed during the experiment in the 5-HT determinations of whole blood is probably of no practical importance But in studies of the effect of 5-HT infusion on the 5 HT amount of the plasma the 5 HIAA may essentially increase the otherwise weak fluorescence of the sample

One of the advantages of the chemical determination is that the fluorescence of 5 HT is directly proportionate to amine concentrations over a very wide range (Sandler 1963) The extinction phenomenon does not emerge until the concentration exceeds 60—70 µg/ml (Sandler, l c) Even the present measurements showed that in the sphere of operation of the present study the 5 HT concentration and intensity of fluorescence were in direct proportion

B DEGRADATION OF 5-HT IN THE LIVER AND IN EXTRAPORTAL CIRCULATION

It is not known how effectively the liver in vivo is capable of controlling the passage of 5-HT by the portal route into extraportal circulation Nor is it known to what extent the organism simultaneously uses mechanisms other than the liver to inactivate 5 HT It was already noted from individual animals associated with the present study*) that the rat liver in certain phases of the experiment, could remove 5 HT from the blood at a rate equalling that of endoportal administration

1 *The porta-hepatic gradient of endogenous 5-HT*

To study the porta hepatic gradient of endogenous 5 HT control samples were drawn from the portal vein and hepatic vein of 35 experimental animals before starting the 5 HT infusion The samples were taken under experimental conditions with the rats under light general anaesthesia and the abdominal cavity opened

Table 2 Contents of endogenous 5 HT in portal vein (P) and in hepatic vein (H) Mean differences of 5 HT contents between matched pairs (P H)

Number of rats	Mean body weight (g) ± SEM	5 HT in P µg/ml Mean ± SEM	5 HT in H µg/ml Mean ± SEM	Mean diff between matched pairs (P H) µg/ml Mean ± SEM
35	231±5 63	1 22±0 04	1 12±0 04	0 11±0 02 $p < 0\ 001$

*) Heikkinen et al Acta Chir Scand. 1967 *133* 475—477

The results are given in Table 2 The portal vein sample contained on an average, 0 11 μg/ml more 5 HT than the hepatic vein sample $(p > 0\ 001)$

2 *Endoportal control infusion with physiological saline 0 2 ml/min*

A control perfusion test was carried out on 12 rats using physiological saline solution The rate of endoportal infusion was 0 2 ml/min The control sample taken before the beginning of perfusion was always drawn from the hepatic vein Furthermore, a total of 4—6 samples were aspirated during perfusion from the different veins, the fewest from the inferior vena cava The perfusion lasted 20—40 minutes

The rats mean body weight was 271 ± 5 33 g The mean weight of liver immediately after the experiment was 8 474 ± 0 414 g Their blood pressures were always recorded, while the pulse ECG and portal pressure were included in only two experiments No essential changes were noted during the perfusion

The portal vein samples were indicated with P, the hepatic vein samples with H and those of the inferior vena cava with C

The samples were divided into five groups according to the moment of aspiration

(1) Control samples drawn before the infusion (H_0 and P_0),
(2) Samples drawn 1—10 min after the start of the infusion (H_1 P_1 and C_1)
(3) Samples drawn 11—20 min after the start of the infusion (H P , and C),
(4) Samples drawn 21—30 min after the start of the infusion (H_3, P_3 and C_3)
(5) Samples drawn 31—40 min after the start of the infusion (H_4 P_4 and C_4)

The infusion of physiological saline and drawing of blood samples did not essentially affect the 5 HT contents of the different veins (Fig 6) The porta hepatic gradient of 5 HT diminished perceptibly during infusion and was no longer of statistical significance The 5 HT contents of the portal and hepatic veins fell slightly as the perfusion time was extended and larger numbers of samples were drawn In the hepatic vein for example the mean relative contents (± SEM) were

$H_0 = 1.00 \pm 0$ ($n^* = 12$), $H_1 = 0.98$ ($n = 2$) $H_2 = 0.98 \pm 0.04$ ($n = 10$) $H_3 = 0.96 \pm 0.03$ ($n = 5$), and $H_4 = 0.92 \pm 0.03$ ($n = 10$) The difference between the initial values and the means of the last samples was statistically almost significant ($p < 0.05$) The aspiration of blood and physiological saline infusion did not seem to reduce the 5 HT contents of the inferior vena cava Admittedly, the number of the samples was so small (3—6) that conclusions are hardly possible On the whole, the 5 HT gradient between the H and C samples was not statistically significant Only the gradient of the H_4 and C_4 samples differed almost significantly from the hypothetic zero value ($p < 0.05$)

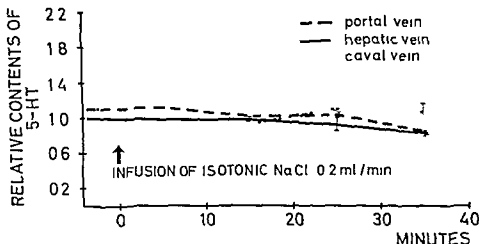


Fig 6 Endoportal infusion of physiological saline 0.2 ml/min Mean values of the relative 5 HT contents and the standard errors of the mean (SEM) with $H_0 = 1.00$ Twelve animals The mean recorded values of P_1 and H_1 are entered at 5 min of P_2 H_2 and C at 15 min of P_3 H_3 and C_3 at 25 min and of P_4 H_4 and C_4 at 35 min A similar method has been applied in all graphs of this type except Fig 8 Numbers of the observation values (n) obtained on the animals and used to calculate the means

H_0 n = 12
P_1 n = 1 H_1 n = 2
P_2 n = 10 H n = 10 C_2 n = 2
P_3 n = 3 H_3 n = 5 C_3 n = 3
P_4 n = 7 H_4 n = 10 C_4 n = 6

3 *Endoportal 5-HT infusion of short duration, 15 µg/min*

In the early phases of the study, 5 HT infusions were maintained for only 10—12 min During this period, samples were drawn at short intervals both from the portal and the hepatic veins No blood pressure

*) n = number of samples and animals

The results are given in Table 2 The portal vein sample contained, on an average, 0 11 μg/ml more 5-HT than the hepatic vein sample ($p > 0\ 001$)

2 *Endoportal control infusion with physiological saline 0 2 ml/min*

A control perfusion test was carried out on 12 rats using physiological saline solution The rate of endoportal infusion was 0 2 ml/min The control sample taken before the beginning of perfusion was always drawn from the hepatic vein Furthermore a total of 4—6 samples were aspirated during perfusion from the different veins the fewest from the inferior vena cava The perfusion lasted 20—40 minutes

The rats mean body weight was 271 ± 5 33 g The mean weight of liver immediately after the experiment was 8 474 ± 0 414 g Their blood pressures were always recorded, while the pulse, ECG and portal pressure were included in only two experiments No essential changes were noted during the perfusion

The portal vein samples were indicated with P the hepatic vein samples with H and those of the inferior vena cava with C

The samples were divided into five groups according to the moment of aspiration

(1) Control samples drawn before the infusion (H_0 and P_0)
(2) Samples drawn 1—10 min after the start of the infusion (H_1 P_1 and C_1)
(3) Samples drawn 11—20 min after the start of the infusion (H_2, P_2, and C_2),
(4) Samples drawn 21—30 min after the start of the infusion (H_3 P_3 and C_3)
(5) Samples drawn 31—40 min after the start of the infusion (H_4, P_4 and C_4)

The infusion of physiological saline and drawing of blood samples did not essentially affect the 5 HT contents of the different veins (Fig 6) The porta hepatic gradient of 5 HT diminished perceptibly during infusion and was no longer of statistical significance The 5 HT contents of the portal and hepatic veins fell slightly as the perfusion time was extended and larger numbers of samples were drawn In the hepatic vein for example the mean relative contents (± SEM) were

Table 3 Endoportal infusion of 5 HT 15 µg/min Its effect on 5 HT contents in portal (P) and hepatic (H) veins Differences (P H) of 5 HT contents of blood between matched pairs before and during infusion of 5 HT

Sample drawn	P Mean relative contents of 5 HT ± SEM in portal vein	H Mean relative contents of 5 HT ± SEM in hepatic vein	Mean P H ± SEM	Significance
Before infusion	1 15±0 03 (15)	1 00±0 00 (15)	0 15±0 03 (15)	p<0 001
During infusion 15 µg/min 0—3 min	2 11±0 14 (13)	—	—	—
During infusion 15 µg/min 4—6 min	2 67±0 23 (5)	1 40±0 09 (13)	1.25±0 33 (5)	p<0 05
During infusion 15 µg/min 7—9 min	2 69±0 40 (11)	1 72±0 08 (9)	1 49±0 53 (6)	p<0 05
During infusion 15 µg/min 10—12 min	2 63 (1)	1 54±0 05 (7)	—	—

Number of rats in brackets | Number of matched pairs in brackets

to be nearly ten times the gradient prior to the infusion The means of relative 5 HT contents of the portal vein were significantly higher than those of the hepatic vein drawn during the corresponding periods

4 *Endoportal 5 HT infusion, 5 µg/min*

5 HT in physiological saline solution was infused into the me enterial vein of six rats at the rate of 5 µg/min The rats mean weight was 258 ± 2 31 g and that of their livers 10 247 ± 0 504 g The control sample was drawn from the hepatic vein Furthermore during each perfusion H P and C• samples as well as H_1 P_1 and C_1 samples were drawn

The results are given in the form of a graph, in Fig 8, with the value of P• entered as 1 11 on the basis of the mean P H gradient of endogenous 5 HT as described above

control was used, but the experiment was discarded if appreciable extravasation occurred These infusions of short duration were given at a rate of 15 µg/min

The group consisted of 15 rats, body weight 225 ± 6 03 g The livers of only six rats were weighed the mean weight obtained was 8 013 ± 0 467 g

For the analysis of these perfusion experiments of short duration, the samples were classified at short time intervals, unlike the other perfusion experiments or groups

P_0 and H_0 control samples of the portal and hepatic veins,
P_1 and H_1 samples drawn 1— 3 min after the start of infusion,
P and H » » 4— 6 » » » » » »
P_3 and H_3 » » 7— 9 » » » » » »
P_4 and H_4 » » 10—12 » » » » » »

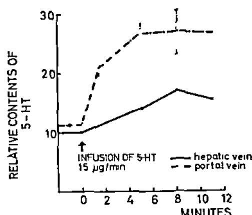


Fig 7 Endoportal 5 HT infusion 15 µg/min Fifteen animals Means of the relative 5 HT contents and their standard errors with H_0 = 1 00
Numbers of the observation values (n) obtained on the animals and used to calculate the means
P_0 n = 15 H_0 n = 15
P_1 n = 13
P n = 5 H_2 n = 13
P_3 n = 11 H_3 n = 9
P_4 n = 1 H_4 n = 7

The results showed that the 5 HT contents of the portal and hepatic veins ceased to increase at 7—9 minutes despite continuing infusion The 5 HT contents of the hepatic vein even showed signs of declining (Fig 7 Table 3) The range of variation in the 5 HT content of the portal vein was as usual during endoportal 5-HT infusion, clearly wider than in that of the hepatic vein or inferior vena cava

Samples from the two veins were seldom drawn from any one rat within the same 3 minute period For this reason matched pairs were too few for determining the significance of the porta hepatic (P-H) 5 HT gradient During 5 HT infusion however this gradient rose steeply so as

Table 3 Endoportal infusion of 5 HT 15 μg/min Its effect on 5 HT contents in portal (P) and hepatic (H) veins Differences (P H) of 5 HT contents of blood between matched pairs before and during infusion of 5 HT

	Sample drawn	P Mean relative contents of 5 HT ± SEM in portal vein	H Mean relative contents of 5 HT ± SEM in hepatic vein	Mean P H ± SEM	Significance
	Before infusion	1 15±0 03 (15)	1 00±0 00 (15)	0 15±0 03 (15)	p <0 001
During infusion 15 μg/min	0—3 min	2 11±0 14 (13)	—	—	—
	4—6 min	2 67±0 23 (5)	1 40±0 09 (13)	1 25±0 33 (5)	p <0 05
	7—9 min	2 69±0 40 (11)	1 72±0 08 (9)	1 49±0 53 (6)	p <0 05
	10—12 min	2 63 (1)	1 54±0 05 (7)	—	—

Number of rats in brackets | Number of matched pairs in brackets

to be nearly ten times the gradient prior to the infusion The means of relative 5 HT contents of the portal vein were significantly higher than those of the hepatic vein drawn during the corresponding periods

4 *Endoportal 5 HT infusion, 5 μg/min*

5 HT in physiological saline solution was infused into the mesenteral vein of six rats at the rate of 5 μg/min The rats mean weight was 258 ± 2 31 g and that of their livers 10 247 ± 0 504 g The control sample was drawn from the hepatic vein Furthermore during each perfusion H P and C samples as well as H_4 P_4 and C_4 samples were drawn

The results are given in the form of a graph in Fig 8, with the value of P_0 entered as 1 11 on the basis of the mean P-H gradient of endogenous 5 HT as described above

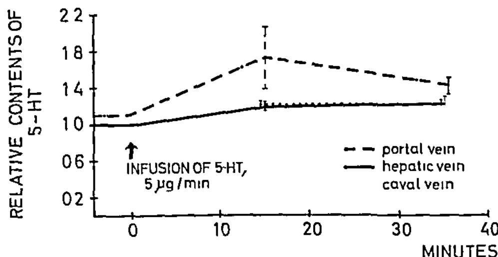


Fig 8 Endoportal 5 HT infusion 5 µg/min Six animals Means of the relative 5 HT contents and their standard errors with H_0 = 1 00
Numbers of the observation values (n) obtained on the animals and used to calculate the means

H_0 n = 6
P_ n = 6 H n = 6 C n = 6
P_4 n = 6 H_4 n = 6 C_4 n = 6

The graph reveals that 5 µg/min of 5 HT produced a rise in the 5-HT content of all the veins The gradients H H_0 and H_4 H_0 differed significantly from the hypothetic zero value ($p < 0\ 001$ and $p < 0\ 01$) The mean rise in extraportal circulation was 19—22 per cent and in the portal vein some 50 per cent Although the portal vein samples in the experiment with one exception, contained definitely more 5 HT than the hepatic vein samples drawn during the same 10 minute period the P H gradient of 5 HT according to the statistical method used was not significant

The 5 HT contents of the H and C samples were very close to each other Usually the serotonin contents of the blood did not change at all on the extraportal circulation side after the first 10—20 minutes although constant 5-HT infusion continued

5 *Endoportal 5 HT infusion, 10-20 µg/min*

10 µg/min of 5 HT was infused endoportally into 11 rats (Group a) and 20 µg/min into 12 rats (Group b) Control samples were usually drawn in these groups both from the portal vein and the hepatic vein

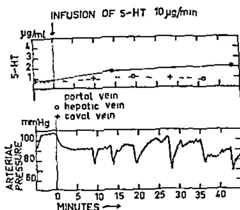


Fig 9 Rat No 82 Endoportal 5 HT infusion 10 µg/min Influence of the infusion on the 5 HT contents of th portal vein hepatic vein and inferior vena cava *and on arterial pressure*

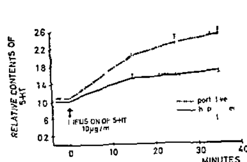


Fig 10 Endoportal 5 HT infusion 10 µg/min Eleven animals Means of the relative 5 HT contents and their standard errors during the infusion with $H_0 = 1\ 00$
Numbers of the observation values (n) obtained on the animals and used to calculate the means
P_0 n = 8 H_0 n = 11
P_1 n = 3 H_1 n = 4
P_2 n = 10 H n = 10 C_2 n = 2
P_3 n = 6 H_3 n = 6 C_3 n = 6
P_4 n = 3 H_4 n = 5

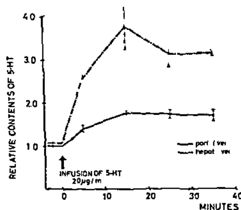


Fig 11 Endoportal 5 HT infusion 20 µg/min Twelve animals Means of the relative 5 HT contents and their standard errors prior to and during the infusion with $H_0 = 1\ 00$
Numbers of the observation values (n) obtained on the animals and used to calculate the means
P_0 n = 8 H_0 n = 12
P_1 n = 8 H_1 n = 8
P_2 n = 10 H n = 12
P_3 n = 3 H_3 n = 6
P_4 n = 2 H_4 n = 5

prior to starting the infusion The perfusions lasted a slightly shorter time than usual average 30 min The fluctuations in the 5 HT contents were mainly observed only in the portal vein and the hepatic vein

In Group a the endogenous 5 HT gradient between the portal vein and the hepatic vein was highly significant ($p < 0\ 001$) whereas in Group b

this porta hepatic difference between the control values lacked statistical significance, although the difference $P_0 H_0$ averaged 0 07 µg/ml the same as in Group a

The rats of Group a had a mean weight of 290 ± 9 340 g and those of Group b 313 ± 3 20 g The rats of Group b were larger than the animals generally employed in the present study

The changes found in the serotonin contents of the blood in both groups during the perfusion tests are shown in Figs 9, 10 and 11 In Group a, the 5-HT contents of the hepatic vein increased by 52—68 per cent and in Group b by 77—71 per cent In the portal veins the corresponding increases were 120—140 per cent and 270—210 per cent The porta hepatic 5-HT gradients of all matched pairs during the infusion differed significantly or highly significantly from the zero value, whereas no distinct difference existed between the hepatic vein and the inferior vena cava In both groups the 5 HT contents of the blood increased during the first ten minutes Subsequently the 5 HT contents hardly changed at all in extraportal circulation

6 *Endoportal and extraportal 5-HT infusion, and endoportal 5 HT infusion following partial hepatectomy, 40 µg/min*

In this group the changes in the 5 HT contents of the blood were studied

(a) during endoportal 5 HT infusion with intact liver
(b) during extraportal 5 HT infusion, and
(c) during endoportal 5 HT infusion after liver resection

(a) Endoportal 5-HT infusion with intact liver

Endoportal infusion of 5 HT was carried out on 17 rats (mean body weight 277 ± 3 20 g) with intact livers, at the rate of 40 µg/min The mean weight of the livers was 9 570 ± 0 330 g

The control samples were drawn from the hepatic vein Infusion lasted 30—40 minutes During the experiment, the control sample plus six samples were usually drawn

The results are given in Figs 12 and 13 The 5 HT contents again increased during the first 10 minutes The subsequent minor changes were of no statistical significance ($p > 0.05$ between the mean values)

The differences (P H) between samples P and H (n=12) and P_4 and H_4 (n = 9) drawn from any one rat during the same ten minute period averaged 3 56 ± 0 70 µg/ml and 2 36 ± 0 47 µg/ml ($p < 0.001$)

On the other hand no essential difference resulted during 5 HT infusion between the 5 HT contents of the hepatic vein and the inferior vena cava The rise produced by infusion in the hepatic vein was 71—80 per cent and in the inferior vena cava 60—72 per cent

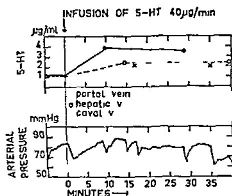


Fig 12 Rat No 130 Endoportal 5 HT infusion 40 µg/min Influence on the 5 HT contents of the different veins and on arterial pressure

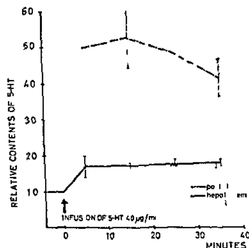


Fig 13 Endoportal 5 HT infusion 40 µg/min Seventeen animals Means of the relative 5 HT contents and their standard errors during the experiment with H_0 = 1 00

Numbers of the observation values (n) obtained on the animals and used to calculate the means

	H_0 n = 17	
P n = 1	H_1 n = 3	
P_2 n = 12	H_2 n = 17	C n = 11
P_3 n = 2	H_3 n = 6	C_3 n = 3
P_4 n = 9	H_4 n = 17	C_4 n = 10

The H C of the matched samples averaged 0 08 ± 0 07 µg/ml and H_4 C_4 0 09 ± 0 08 µg/ml ($p > 0\ 05$) These gradients suggested a slight extrahepatic degradation of 5 HT

(b) Extraportal 5-HT infusion

5 HT was infused through the jugular vein directly into the extraportal circulation of six animals Their mean weight was 277 ± 6 53 g and the mean weight of their livers 9 040 ± 0 570 g

The porta hepatic 5 HT gradient was considerably smaller than in Group (a) The $P_2 H_2$ averaged 0 72 ± 0 08 µg/ml ($p < 0\ 001$), and $P_4 H_4$ was 0 52 ± 0 10 µg/ml ($p < 0\ 01$)

Table 4 Effect of 5 HT infusion route on relative 5 HT contents in blood

	Time of sample drawn and the vein involved	Mean relative contents of 5 HT ± SEM		Diff between the two means ± SEM and signif
		Portal infusion	Jugular infusion	
	Before infusion H	1 00±0 (17)	1 00±0 (6)	±0
During infusion 40 µg/min	11—20 min P_2 *)	5.29±0 68 (12)	3 63±0 22 (6)	+1 66 µg/ml $p > 0\ 05$
	31—40 min P_4 *)	4 14±0 41 (9)	3 51±0 19 (6)	+0 63 µg/ml $p > 0\ 05$
	11—20 min H_2 *)	1 72±0 06 (17)	2 91±0 16 (6)	−1 19 µg/ml $p < 0\ 001$
	31—40 min H_4 *)	1 80±0 08 (17)	2 99±0 22 (6)	−1 19 µg/ml $p < 0\ 001$
	11—20 min C *)	1 60±0 04 (11)	3 24±0 16 (6)	−1 64 µg/ml $p < 0\ 001$
	31—40 min C_4 *)	1 66±0 08 (10)	3 40±0 16 (6)	−1 74 µg/ml $p < 0\ 001$

) P=portal vein H=hepatic vein and C=inferior vena cava

Number of samples and number of rats in brackets

Table 4 compares the differences found on using two different routes of infusion Administration of serotonin directly into extraportal circulation developed a very much more pronounced hyperserotoninaemia within the region of the extraportal circulation than did administration *of serotonin into the organism through the liver*

During a 5 HT infusion through the jugular vein the 5-HT contents of the portal vein and the inferior vena cava were approximately equal.

(c) Endoportal 5-HT infusion after partial hepatectomy

The left lateral lobe of the liver was resected, using the technique described by Higgins and Andersen (1931), in six rats taking care that their arterial pressure was not lowered due to the resection. In a further ten animals, both the left lateral and the middle lobes of approximately the same weight, were resected. Each lobe represented roughly one third of the liver's weight.

After resection the aspiration and infusion needles were placed in position and a control sample was drawn from the hepatic vein. Subsequently the usual perfusion test was performed by infusing 5-HT endoportally at the rate of 40 µg/min.

Table 5 Resection of left lateral lobe of the liver (one third hepatectomy) and its effect on degradation of endoportally infused 5 HT, 40 µg/min

Time of sample drawn and vein involved		Mean relative contents of 5 HT ± SEM		Differences between the two means and their significance
		5 HT infusion with intact liver	5 HT infusion after resection	
Before infusion H_0)		1.00±0 (17)	1.00±0 (6)	±0
During infusion 40 µg/min	11—20 min H	1.71±0.24 (17)	2.38±0.28 (6)	0.67 $p<0.01$
	21—30 min H_3	1.77±0.12 (6)	2.93±0.50 (5)	1.16 $p<0.05$
	31—40 min H_4	1.80±0.08 (17)	3.09±0.39 (6)	1.29 $p<0.001$

Number of rats in brackets

) H = hepatic vein

Table 5 shows the percentages of 5 HT change in the hepatic vein in experiments after resection of one third of the liver compared with the results obtained on perfusion of intact liver. In this group the animals

wighed 285 ± 8 85 g, and the remaining part of the liver weighed 6 680 ± 0 390 g During the infusions, the amount of 5 HT was significantly larger in the extraportal circulation of the resected animals than of those with intact liver

Table 6 Resection of left lateral and median lobes of the liver (two thirds hepatectomy) and its effect on degradation of endoportally infused 5 HT 40 µg/min

Time of sample drawn and vein involved		Mean relative contents of 5 HT ± SEM		Diff between the two means µg/ml and their signif
		Infusion of 5 HT with intact liver	Infusion of 5 HT after resection	
Before infusion H_0 *)		1 00±0 (17)	1 00±0 (10)	0±
During infusion 40 µg/min	11—20 min H *)	1 71±0.24 (17)	5 06±0 61 (8)	3 35 $p<0\ 001$
	21—30 min H_3 *)	1 77±0 12 (6)	5 07±0 73 (8)	3 30 $p<0\ 01$
	31—40 min H_4 *)	1 80±0 08 (17)	5 04±0 30 (9)	3.24 $p<0\ 001$

Number of samples and number of rats in brackets

) H hepatic vein

Table 6 correspondingly, shows the effect of the resection of two-thirds *of the liver on the degradation of 5 HT given endoportally The* 5 HT contents in the hepatic vein increased to five times the control level While the increase in the group with perfusion of the intact liver was only 75 per cent, it was 405 per cent in these animals

In the animals subjected to a resection of two thirds of the liver, an almost significant difference in 5 HT contents was noted also between *the hepatic vein and the inferior vena cava e g H C* (seven matched pairs) was 1 14 ± 0 41 µg/ml ($p<0\ 05$) Hence the degradation of 5 HT in the extraportal circulation was also considerable

8 *Endoportal 5-HT infusion, 80 µg/min*

There were 26 animals in the group with a mean weight of 268 ± 3 90 g The mean weight of the liver was 8 830 ± 0 160 g

Fig 14 shows a graph of the mean relative 5 HT contents in the different veins with an initial value (H_0) of 1 00

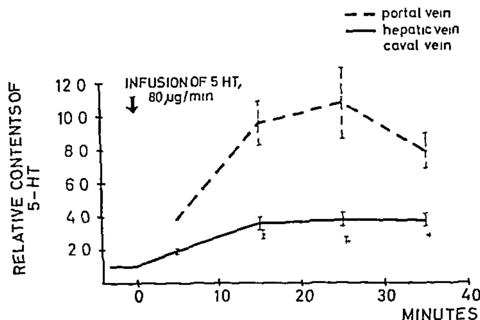


Fig 14 Endoportal 5 HT infusion 80 µg/min Twenty six animals Means of the relative 5 HT contents and their standard errors during the experiment with $H_0 = 1\ 00$ Numbers of the observation values (n) obtained on the animals and used to calculate the means

	H_0 n = 26	
P_1 n = 2	H_1 n = 3	
P_2 n = 18	H_2 n = 24	C_2 n = 15
P_3 n = 4	H_3 n = 17	C_3 n = 6
P_4 n = 12	H_4 n = 25	C_4 n = 17

This group fell into two subgroups in some rats the extraportal blood 5 HT content remained reasonably low despite the continuing 5 HT infusion while in others it did not In neither subgroup did the 5 HT content of the blood change essentially after 11—20 min The 5 HT gradient between prehepatic and posthepatic blood in the whole group

Table 7 Endoportal infusion of 5-HT 80 µg/min and its effect on contents of 5-HT in the blood. Porta hepatic (P-H) and hepatocaval (H-C) 5-HT differences between matched pairs during infusion of 5-HT

Sample drawn		Mean 5-HT ±SEM in portal vein (P)	Mean 5-HT ±SEM in hepatic vein (H)	Mean 5-HT ±SEM in caval vein (C)	Mean diff ± SEM between P and H	Mean diff ± SEM between H and C
Before infusion		—	1.00±0.00 (26)	—	—	—
During infusion 80 µg/min	11—20 min	9.06±1.29 (18)	3.59±0.38 (24)	2.92±0.26 (15)	4.46±1.29 (16) $p<0.01$*)	1.07±0.36 (14) $p<0.01$*)
	31—40 min	7.84±1.02 (12)	3.76±0.36 (25)	2.87±0.27 (17)	4.52±0.95 (12) $p<0.001$*)	0.79±0.22 (17) $p<0.01$*)

Number of rats in bracket

Number of matched pairs in brackets

*) Significance of the differences from zero

was 4.50 and that between the hepatic vein and the inferior vena cava was ca. 1.00. These gradients and their significance are shown in Table 7.

80 µg/min of 5-HT, given endoportally produced a significant H-C gradient. With smaller endoportal doses, no distinct 5-HT gradient could be noted between the hepatic vein and the inferior vena cava on perfusion of the intact liver as described above.

For arterial pressure responses this group was similarly divided into two subgroups (see p. 45). In some animals the 5-HT infusion produced only a monophasic depressor response, in others a triphasic response. It was found that the rats, in which the extraportal blood 5-HT content remained reasonably low during the infusion, showed a monophasic response to serotonin infusion (Table 8).

Only in extraportal circulation were the differences in 5-HT contents statistically significant between the two subgroups. The gradient of matched pairs between the hepatic vein and the inferior vena cava (H-C) was definitely different in the different subgroups. In animals with triphasic arterial pressure response the H-C ($n = 11$) was 1.50

Table 8 Endoportal infusion of 5 HT 80 µg/min Difference of its effect on blood 5 HT between rats with monophasic and triphasic response of arterial blood pressure

Time of sample drawn and vein involved		Mean relative contents of 5 HT ± SEM — Arterial pressure response: Monophasic	Mean relative contents of 5 HT ± SEM — Arterial pressure response: Triphasic	Differences between the two means and their signif
Before infusion H_0		1 00±0 (11)	1 00±0 (15)	±0
During infusion 80 µg/min	11—20 min P)	7 70±2 81 (5)	9 58±1.30 (13)	1 88 p>0 05
	31—40 min P_4)	7 58±2.28 (5)	10 20±1 02 (7)	2 62 p>0 05
	11—20 min H *)	2 09±0 15 (10)	4 67±0 46 (14)	2 58 p<0 001
	31—40 min H_4)	2 88±0 29 (11)	4 46±0 53 (14)	1 58 p<0 05
	11—20 min C *)	1 90±0 11 (5)	3 40±0.29 (10)	1 50 p<0 01
	31—40 min C_4)	2 29±0 30 (7)	3 24±0 23 (10)	0 95 p<0 05
Body weight g ± SEM		274±6.30	264±4 66	10 g
Liver weight g ± SEM		9 090±0 380	8 630±0.270	0 460 g (p<0 05)

Number of rats in bracket
*) P=portal vein H=hepatic vein C=caval vein

± 0 41 µg/ml (p<0 01) while the corresponding difference in the other subgroup (n = 4) was only 0 22 ± 0 19 µg/ml (p> 0 05)

The livers of the animals with triphasic arterial pressure response weighed more than those of the animals with a monophasic response, as can be seen from Table 8

9 *Endoportal 5-HT infusion, 160 µg/min*

There were 11 animals in the group their mean weight being 258 ± 4 74 g The mean weight of the liver was 8 630 ± 0 390 g

The effect of an endoportal infusion of 5 HT on the 5-HT content of the blood is shown in Fig 15

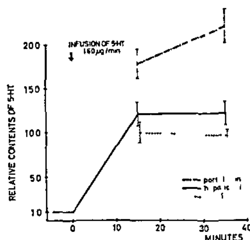


Fig 15 Endoportal 5 HT infusion 160 µg/min Eleven animals Means of the relative 5 HT contents and their standard errors (SEM) during the experiment with $H_0 = 1\ 00$
Numbers of the observation values (n) obtained on the animals and used to calculate the means
H_0 n = 11
P n = 9 H_2 n = 9 C n = 10
P_4 n = 9 H_4 n = 10 C_4 n = 11

During the experiments a strong spasm was occasionally seen in the mesenterial vein into which the infusion was given The vascular spasm apparently produced uneven endoportal dosage of 5 HT, manifested in a remarkable fluctuation of the P values The peristalsis of the bowel was so pronounced that the termometer if unfixed, was expelled from the rectum In the first ten minutes the excursion of respiratory movement and the amplitude of the pulse were small despite constant artificial respiration and arterial hypertension

As can be seen from Fig 15 the main changes in the 5-HT contents of the blood had occurred by the time the 10—20 minute samples were drawn Subsequently the situation was practically constant, and both the porta hepatic (P H) and hepato caval (H C) gradients of the 5-HT were prominent These differences in serotonin contents in matched pairs (samples taken from the same animals during the same 10 minutes) were

Porta hepatic gradient

P H (n = 6) = 5 82 ± 2 50 ($p < 0\ 05$)

P_4 H_4 (n = 8) = 8 20 ± 1 23 ($p < 0\ 001$)

and hepato caval gradient

H C (n = 8) = 2 23 ± 0 82 ($p < 0\ 05$)

H_4 C_4 (n = 10) = 3 17 ± 0 82 ($p < 0\ 01$)

It is seen that the porta hepatic 5 HT gradient was roughly 2 5 times the hepato caval gradient

10 *Endoportal 5-HT infusion, 320 µg/min*

The group consisted of six rats their mean weight was 283 ± 1 17 g and the mean weight of their livers 9 850 ± 0 370 g

The animals tolerated even this rate of infusion relatively well The first 10—20 minutes seemed to be worst, as in the earlier experiments Aspiration of blood reduced the arterial pressure in this group more readily below the permissible minimum limit (60 mmHg) than in the groups treated with a rate of infusion below 320 µg/min

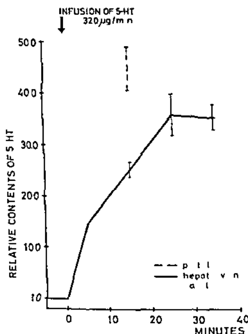


Fig 16 Endoportal 5 HT infusion 320 µg/min Six animals Means of the relative 5 HT contents and their standard errors (SEM) during the experiment with H_0 = 1 00
Numbers of the observation values (n) obtained on the animals and used to calculate the means
H_0 n = 6 H_1 n = 1
P_2 n = 5 H n = 6 C_2 n = 5
H_3 n = 3
H_4 n = 6

The mean relative 5 HT contents seen during the infusion in the different veins and their standard errors are plotted in Fig 16 Unlike the preceding series the 5 HT contents now continued to increase in many cases throughout the experiment

The porta hepatic 5 HT gradient of matched pairs was

P H (n = 6) = 16 92 ± 4 60 (p <0 05)

P_4 H_4 (n = 3) = 13 56 ± 1 33 (p <0 01)

and the hepato caval amine gradient

H C (n = 6) = 5 29 ± 1 84 (p<0 01)

Consequently, the transhepatic difference in serotonin contents was three time the difference recorded when the blood passed throuh the lungs and the distal half of the body to the inferior vena cava

11 *Effect of endoportal 5-HT infusion on the 5-HT contents of the plasma*

ɔ-HT was infused endoportally into six rats at the rate of 40 µg/min The mean weight of the rats was 278 ± 11 3 g that of their livers 9 970 ± 0 400 g Samples were taken only from the portal and hepatic veins The 5 HT contents of the plasma before and during infusion are given in Table 9 The table also shows the results of the three

Table 9 Effect of endoportal infusion of 5 HT on 5 HT contents in plasma

Time of sample drawn and vein involved		Mean contents of 5 HT in plasma µg/ml ± SEM		
		Endoportal inf of 5 HT 40 µg/min	Endoportal inf of 5 HT 80 µg/min	Endoportal inf of 5 HT 160 µg/min
Before infusion H₀)		0 03±0 01 (6)	—	—
During infusion	11—20 min P₂ *)	1 68±0 31 (5)	4 34±0 70 (3)	—
	31—40 min P₄)	3 88±1 94 (5)	13 14±4 94 (3)	—
	11—20 min H₂ *)	0 19±0 09 (6)	0 18±0 08 (3)	7 86 (2)
	31—40 min H₄)	0 29±0 09 (6)	0 84±0 42 (3)	9 03 (2)

Number of samples and number of rats in brackets
) H=hepatic vein P=portal vein

experiments with an infusion rate of 80 µg/min and the two experi ments with an infusion rate of 160 µg/min

It is seen from the table that the plasma 5 HT contents in extraportal circulation remained low when 5 HT was infused endoportally at the

rate of 40 or 80 μg/min, whereas the two rats treated with infusions at the rate of 160 μg/min showed very high 5-HT contents also in the extraportal plasma during infusion

Discussion

The result according to which the portal vein contains more 5-HT than the hepatic vein reveals that some 5 HT disappears from the blood during its transhepatic circulation It also reinforces the view that 5 HT is released into the blood by the intestinal argentaffin cells (Toh 1954, Erspamer and Testini 1959)

The porta hepatic endogenous 5 HT gradient now recorded in the rat has previously been demonstrated in the dog (Toh 1954, Drabanas and McDonald 1963) Toh (1954) reports the 5-HT difference between the prehepatic and posthepatic blood at 0 09 μg/ml, and Drabanas with his co-workers (1963) at 0 07 μg/ml The rats porta-hepatic 5 HT gradient of 0 11 μg/ml implies a liver clearance of 5-HT of only 9 per cent while the 0 07 μg/ml obtained for dogs corresponds to a liver clearance of 30 per cent (Drabanas and McDonald 1963)

Various stimuli are known to increase the release of 5 HT from the intestinal tract (O Hara et al 1959 Karki et al 1960 Drabanas et al 1962, Peskin and Miller 1962, Warner et al 1966a b) Since such factors of intestinal action may be present in laparotomy conditions stimulating the release of serotonin it is quite possible that the porta-hepatic gradient of rats under physiological conditions is smaller than the gradient now recorded

The 5 HT of the blood is mainly contained in the thrombocytes (Rand and Reid 1951, Humphrey and Toh 1954) Only minute amounts of endogenous 5 HT are free in the plasma (Toh 1954, Collier 1958, Crawford 1966) Apparently only free 5 HT is active and can be metabolized (Erspamer 1961 1966) Therefore the thrombocytes when they absorb the free serotonin from the plasma at the same time inactivate store and transport it safely through enzyme-containing organs from one site to the other

The 5 HT content of the plasma is so low that the transhepatic or transpulmonary 5 HT gradients observed now and earlier cannot result from the metabolism of the plasma fraction Furthermore it has been impossible to demonstrate any differences in the 5 HT contents of plasma

between blood entering and leaving these organs (Toh 1954, Erspamer and Testini 1959, Crawford 1965)

Theoretically, the 5 HT of the thrombocytes can be drawn into the sphere of the liver enzymes by two routes through decomposition of thrombocytes and/or release from intact platelets

Serotonin is released in thrombocytolysis (Stacey 1958) No decrease in the number of thrombocytes has been demonstrable, however, in the course of transhepatic (Toh 1954) or transpulmonary (Crawford 1965) circulation while the organs are intact Hence liberation and metabolism of 5-HT through thrombocytolysis apparently does not suffice to account for the porta hepatic gradient obtained

The alkaline extract (nephrosin) of the kidney liver and even of the lungs produces in vitro a liberation of serotonin and histamine from thrombocytes (Toh 1956) But there is no certainty that this tissue principle also acts in vivo (Paasonen 1965)

Opinions on the interchange of the 5 HT in the thrombocytes are controversial Some authors find that thrombocytolysis is the only possibility of 5 HT release (Udenfriend and Weissbach 1958) This stable fixation of molecules in a living organism appears unlikely Erspamer (1961) speaks of stable and unstable intracellular 5 HT, the unstable being readily released if required

Hughes and Brodie (1959) advanced the opinion that there was a dynamic equilibrium in vitro between the 5 HT molecules of plasma and thrombocytes with emphasis markedly on the 5-HT of the thrombocytes The same year, Born and Gillson demonstrated by experiment in vitro that the 5 HT molecules of thrombocytes saturated with 5 HT were very rapidly exchanged with the radioactive 5 HT molecules of the environment The exchange was as rapid as the 5-HT uptake of unsaturated thrombocytes in its initial phase This seems to suggest that very rapid exchange may occur to maintain dynamic equilibrium between plasma and intracellular amine molecules

On the basis of the above it may be assumed that in tissues in which the free 5 HT of the plasma is metabolized rapid outflux of 5 HT molecules from the thrombocytes occurs to maintain the equilibrium On the other hand, where excess 5-HT is released or injected into plasma its influx into the platelets outweighs the outflux until dynamic equilibrium is reached

Environmental conditions according to in vitro studies affect the absorption capacity of the thrombocytes (Weissbach et al 1958a Stacey 1961) and hence the plasma thrombocyte ratio of the 5 HT It may be

assumed, perhaps, that the pH and ion ratios in the liver presupposed a smaller thrombocyte/plasma gradient of 5 HT than those prevailing in extrahepatic conditions with the result that some of the thrombocyte 5 HT was released during intrahepatic circulation

The dynamic equilibrium between the 5 HT molecules of thrombocytes and plasma is probably the most natural explanation for the finding that the liver is able to remove some of the 5 HT of the whole blood without any transhepatic gradient being observable in the 5 HT contents of the plasma

In the present experiments it was seen that during the first few minutes of the endoportal 5 HT infusion the liver removed only some of the 5-HT administered while some of it entered extraportal circulation This was also seen in the results reported by Drabanis (1963) on a 15—20 min perfusion of intact dog liver with 5 HT In the early phase of the experiments therefore, the prehepatic rise in the 5 HT content of the blood markedly affected the posthepatic side This may also explain the negative view Johnson et al (1961) held on the ability of dog s liver to inactivate 5 HT in vivo the duration of the infusion was not reported

The active ability of the platelets to maintain a high 5 HT gradient vis a vis plasma considerably exceeds the normal need (Humphrey and Toh 1954, Hardisty and Stacey 1955) In vitro thrombocytes are capable of practically trebling their serotonin content while the plasma/thrombocyte 5 HT ratio remains within the range 1 200 — 1 1000 (Stacey 1958 Hughes and Brodie 1959) In an in vitro suspension of thrombocytes and plasma equilibrium between the cells and their environment was found to establish itself within about an hour, and in the initial phase the 5 HT uptake by the platelets was very fast (Brodie et al 1957 Stacey 1958 Born and Gillson 1959 Hughes and Brodie 1959) In optimal in vitro conditions the equilibrium was reached within 30 minutes (Burningham et al 1966)

Once the active absorption mechanism of the thrombocytes is saturated with 5 HT the 5 HT content of the thrombocytes increases only in direct proportion to the 5 HT content of the plasma (Hughes and Brodie 1959) The uptake of 5 HT molecules by the thrombocytes after saturation is passive diffusion Reserpine does not affect it although it completely blocks the active transport system (Hughes and Brodie 1959)

According to Stacey (1958) the red cells also absorb some 5 HT Erythrocyte 5 HT increases a least in direct proportion to the rise in plasma 5 HT content through passive diffusion Hence the 5 HT taken up by the red cells may have constituted a remarkable portion of the

inactive intracellular 5 HT fraction in the present perfusion experiments

To study the extent to which the 5 HT inactivation by blood elements was of passive character, a few experiments were carried out on reserpinized animals On the day before the experiment the rats were intraperitoneally given 0 50 mg reserpine per kg of body weight The animals were given 20 µg/min of 5 HT endoportally The 5 HT content of the portal vein was found to increase on an average, from 0 10 to 1 30 µg/ml, and that of the hepatic vein from 0 10 to 0 35 µg/ml The 5 HT of extraportal blood thus rose only by one-third of the rise produced by the corresponding dose in the rats not treated with reserpine This result supports the opinion that the active function of thrombocytes played an important role in the formation of the inactive intracellular 5 HT fraction during infusion experiments

On the basis of the absorptive function of the thrombocytes and possibly of other blood cells as well it is easy to understand why the liver in the early phases of the infusion tests removed only some of the 5-HT administered and why with continued infusion and sufficiently small doses it removed practically all of the 5 HT administered At the beginning of the experiments the blood elements in the first place absorbed an important share of the 5 HT, since their degree of saturation with 5-HT was low After about 10 minutes (Figs 7 and 11) the 5-HT inactivation by thrombocytes and erythrocytes based on 5 HT absorption and diffusion ceased to be visible irrespective of the rate of infusion Apparently by this time a dynamic equilibrium had been reached in the portal vein with the outflux of 5 HT molecules from the cells equal to the influx The larger the amounts of 5 HT administered, the more of it was stored in the intracellular spaces of the blood It therefore follows naturally that extraportal blood showed higher 5 HT contents on infusion of 5 HT at the rate of 40 µg/min than on infusion at 5 µg/min although the liver in both cases was capable of practically 100 per cent inactivation

When 5 HT was injected directly into extraportal circulation at the rate of 40 µg/min it was found that the hepatic vein contained 1 5 times more 5 HT than it did on endoportal infusion at the same rate This can also be explained on the basis of the absorptive function of thrombocytes and erythrocytes

Therefore in the first ten minutes of endoportal 5 HT infusions apparently the blood elements and the liver efficiently prevented the access of free active 5 HT into systemic circulation Once the 5-HT molecules of the plasma and blood elements primarily thrombocytes

had reached equilibrium the liver alone was responsible for the inactivation of the infused 5 HT The liver was capable of achieving this up to an infusion rate of 80 µg/min Not until the dose exceeded this did the 5 HT content of the extraportal blood plasma increase, and only then did the organism start using the inactivation mechanisms of other organs to an extent demonstrable by the method employed in the present study

320 µg/min of 5 HT was such a big dose that the 5 HT contents of blood samples still increased 30 min after the start of the infusion This probably implies that the enzymatic forces of the whole organism were unable to degrade 5 HT from the free 5-HT quantity which remained as substrate, at a rate equalling that of 5 HT administration

The essential part played by the liver in 5-HT metabolism was distinctly seen also in perfusions following partial hepatectomy A successful liver resection did not demonstrably affect the rats haemodynamics This notwithstanding, the organism of the animals subjected to resection of two thirds of the liver and an infusion at the rate of 40 µg/min certainly had to resort to extrahepatic mechanisms of inactivation, since a hepato caval 5 HT gradient could be demonstrated

In extraportal 5-HT infusions at 40 µg/min the blood having passed through the lungs and the splanchnic region into the portal vein, still contained so much free 5 HT that the liver could maintain a 5 HT gradient of 0 5—0 7 µg/ml Assuming that the portal flow during the experiments was of normal magnitude 15 ml/min the liver did remove some 25 per cent of the amine administered

The part played by monoamino oxidases in the 5 HT degradation in the liver was studied by giving the rats 15 mg pheniprazine per kg of body weight endoportally before the 5 HT infusion Preliminary results suggest that MAO inhibition reduced the capacity of the liver to inactivate 5 HT from 80 µg/min to 40 µg/min The inhibition produced was so effective that thin layer chromatography failed to show the 5 HIAA spot in the plasma of the hepatic vein sample (see Fig 5)

Besides liver, the lungs have been considered another important inactivator of 5 HT Abundant evidence has been reported to support this view (Gaddum et al 1953 Goble et al 1955, Eiseman et al 1964 Davis and Wang 1965 Crawford 1965 Moore et al 1966) The present experiments showed that the pulmonary barrier to serotonin is weaker than the hepatic barrier This concurs with the tissue enzyme studies according to which monoamine oxidase activity is considerably greater in the liver than in the lungs (Klingman 1966)

Thrombocytes have been found to metabolize in vitro the 5 HT released from them by reserpine into plasma or tyrode solution (Paasonen 1961, Paasonen and Airaksinen 1965, Pletscher et al 1966) On 3 hour incubation at 37°C of such a thrombocyte plasma suspension of the rats, the loss of 5 HT was only 4 4 per cent that is to say, 0 11 μg/ml (Paasonen 1961) Hence the enzymatic inactivation of thrombocytes is very slow, in any case in rats Since 5 HT, injected or released into the mesenterial vein, travels into systematic circulation in 2 3 seconds such a slow inactivation mechanism could hardly affect the porta-hepatic or hepato caval 5 HT gradients of the present experiments

It has been assumed that the ceruloplasmin of the plasma also might be of importance for minimizing the plasma fraction of 5 HT (O Reilly and Loncin 1967) Speculations on the effect of this oxidation system on 5 HT in vivo are so far completely hypothetic and theoretical

In addition to enzymatic destruction and the absorptive function of the platelets and other blood cells biological inactivation of 5-HT occurs by diffusion into the tissues and by excretion of free 5 HT

According to many authors, the 5 HT does not cross at all e g the blood brain barrier (Erspamer 1961, Garattini and Valzelli 1965) Minute quantities of the pharmacological doses of 5 HT, however, have been found to travel into the brain primarily to its primitive parts (Costa and Aprison 1958 Karki and Paasonen 1959, Tammisto 1965, Bulat and Supek 1957) Bulat and Supek injected a very large dose of 5 HT (10 mg/kg) into the extraportal circulation of some rats and analysed tissue specimens taken after 10 minutes They found that the 5 HT content of the intestine had not increased at all, that of the brain had increased very slightly while in the lungs and the liver the increase was most pronounced The 5 HT content of liver tissue increased by 0 3—1 0 μg/g The large dose producing marked hyperserotoninaemia therefore increased the 5 HT in hole liver only by a few micro grams Furthermore if endoportal infusion of 5 HT is used, only small quantities of serotonin pass into extraportal circulation therefore the diffusion of 5 HT into the tissues is hardly of any importance in the present experiments The removal of free 5 HT from the organism as such also represents a very small percentage even when large pharmacological doses are used (Keglevic et al 1959, Airaksinen 1963)

C CORRELATION OF HAEMODYNAMIC CHANGES TO INACTIVATION OF INFUSED 5 HT

Va omotor disturbances are essential among the symptoms accompanying the carcinoid (McKusic 1956) Numerous studies have been carried out both on humans (Page 1958 Sjoerdsma 1959, Bojs 1961 Erspamer 1961 1966 Robson and Stacey 1962) and on various animal species (Reid 1951, Page and McCubbin 1953 Schneider and Yonkman 1954, Salmoiraghi et al 1956 Outschoorn and Jacob 1960) to analyse the haemodynamic responses to 5-HT administered directly into extraportal circulation Attempts to explain the mechanism of the action of 5 HT on the cardiovascular system by means of the highly varying results obtained have always involved the use of uncertain hypotheses The authors of reviews in fact term the pharmacological in vivo effects of 5 HT on the circulatory system as varying and insufficiently analysed (Robson and Stacey 1962, Garattini and Valzelli 1965, Erspamer 1966) Comments on hyperserotonismus as related to the vasomotor symptoms of the carcinoid syndrome have become reserved (Peart and Robertson 1961) For example, the humoral basis of flushing awaits elucidation and is an issue in dispute (The Lancet, editorial 1966)

1 *Systemic blood pressure*

In connection with the endoportal infusions of 5 HT four types of response were seen in the rats blood pressure recorded from the carotid artery

(1) no effect
(2) monophasic depressor effect
(3) triphasic effect
(4) biphasic effect

Thrombocytes have been found to metabolize in vitro the 5 HT re leased from them by reserpine into plasma or tyrode solution (Paasonen 1961 Paasonen and Airaksinen 1965, Pletscher et al 1966) On 3 hour incubation at 37°C of such a thrombocyte plasma suspension of the rats the loss of 5 HT was only 4 4 per cent, that is to say, 0 11 μg/ml (Paasonen 1961) Hence the enzymatic inactivation of thrombocytes is very slow in any case in rats Since 5 HT, injected or released into the mesenterial vein travels into systematic circulation in 2 3 seconds such a slow inactivation mechanism could hardly affect the porta hepatic or hepato caval 5 HT gradients of the present experiments

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In addition to enzymatic destruction and the absorptive function of the platelets and other blood cells, biological inactivation of 5-HT occurs by diffusion into the tissues and by excretion of free 5 HT

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The biphasic response lacked the initial depressor phase This response began with a sudden arterial pressure rise which then turned into permanent hypotonia

Table 10 The types of systemic blood pressure response to 5 HT infusion by different routes and at different rates into rats with intact liver and partially hepatectomized rats

Rate and route of infusion of 5 HT	Number of rats in the groups of different blood pressure response		
	No effect	Monophasic	Triphasic or biphasic
Endoportal 5 µg/min (6)	6	0	0
Endoportal 40 µg/min (17)	0	16	1
Endoportal 80 µg/min (26)	0	11	15
Endoportal 160 µg/min (11)	0	0	11
Endoportal 320 µg/min (6)	0	0	6
Extraportal 40 µg/min (6)	0	0	6
Endoportal after resection of one third of the liver 40 µg/min (6)	0	2	4
Endoportal after resection of two thirds of the liver 40 µg/min (10)	0	1	9

Number of rats in brackets

The endoportal 5-HT dose of 5 µg/min produced in no case a demonstrable change in arterial pressure (Table 10) Endoportal infusions of 5 HT at the rates of 10—20 µg/min or 40 µg/min produced a monophasic response In only one case (Rat No 169) did infusion at a rate of 40 µg/min produce a slightly triphasic curve The relative 5 HT values recorded in the hepatic vein of this rat were the highest of the group $H_1 = 2\ 18$ H $= 2\ 36$ $H_3 = 2\ 05$ and $H_4 = 2\ 27$

The animals into which 5 HT was infused endoportally at the rate of 80 µg/min fell into two groups by their arterial pressure response as pointed out earlier (p 45) 11 rats showed a monophasic and 15 a triphasic response As can be seen from Table 8, the former group let very limited amounts of 5-HT into the hepatic vein, especially in the initial phase of the infusion In these animals the relative 5 HT contents of the H samples averaged 2 09 ± 0 15 µg/ml (n = 10) while in the rats with triphasic response the corresponding mean contents were significantly larger than those of the former group H = 4 46 ± 0 53 µg/ml (n = 14)

Four rats were given an endoportal infusion of 5 HT at the rate of 120 µg/min They all had a triphasic arterial pressure re ponse

Endoportal infusion of 5 HT at the rate of 160 µg/min also regularly produced a triphasic arterial pressure curve The only difference between that and the curve of the 120 µg/min group was that the hypertensive phase following the initial phase predominated and always rose distinctly above the initial level The permanent hypotensive phase following this hypertensive phase also was more pronounced than that of the rats treated with a 120 µg/min infusion

Infusion at 320 µg/min produced either a pressor dominated triphasic or a simply biphasic pressure curve

On endoportal infusion at a rate of 40 µg/min after resection of one third of the liver four rats showed a triphasic and two a monophasic curve In one of these two, the H sample of the hepatic vein contained only 54 per cent, and in the other 100 per cent more 5 HT than the control sample For the whole group, the mean increase in the 5 HT content of the hepatic vein was 193 per cent

After resection of two thirds of the liver endoportal infusion at 40 µg/min produced either a biphasic or a pressor dominated triphasic blood pressure response Only one rat (No 112) showed monophasic response The first sample was not drawn from the hepatic vein of this rat until perfusion had gone on for 20 min Even then it contained only 133 per cent more 5 HT than the control sample This was the lowest 5 HT content recorded for the hepatic vein during infusion in this group in which the H samples contained an average of 406 per cent more 5 HT than the control samples

When 5 HT was infused directly into extraportal circulation at the rate of 40 µg/min, the arterial pressure curve was always triphasic, the pressure in the hypertensive phase exceeding the initial level

2 *Pulse rate*

Like the blood pressure changes the variations in pulse rate occurred in the initial phase of infusion For this reason the pulse rates were analysed only during the first ten minutes, and compared with the pulse rates recorded immediately before the start of the infusion No blood samples were drawn while pulse rates were analysed

Table 11 Effect of infusion of 5 HT on pulse rate

Groups of rats	Mean pulse/min ± SEM before infusion of 5 HT (=Control p/min)	Mean differences (± SEM) of pulse/min in matched pairs	
		Pulse/min in initial phase *) control p/min subtracted	Pulse/min in final phase control p/min subtracted
Endoportal infusion 5 μg/min	373±2 (6)	0±1 (6)	0±6 (6)
Endoportal infusion 40 μg/min	341±18 (9)	+ 11±4 (7)	+ 13±19 (8)
Endoportal infusion 80 μg/min Monophasic arterial pressure response	384±17 (8)	+ 10±3 (5)	− 3±8 (7)
Endoportal infusion 80 μg/min Triphasic arterial pressure response	372±22 (7)	− 68±25 (6)	+ 102±9 (5)
Endoportal infusion 160 μg/min	391±4 (7)	− 21±11 (7)	+ 107±45 (7)
Endoportal infusion 320 μg/min	360±6 (3)	− 193±47 (3)	+ 100±30 (3)
Extraportal infusion 40 μg/min	323±17 (6)	− 42±9 (6)	+ 152±15 (6)

Number of rats in brackets

*) 0—2 min after beginning of 5 HT infusion

Number of matched pairs of pulse in brackets

As reported above with large doses the arterial pressure response was triphasic (or biphasic) The initial depressor phase lasted for a maximum of 2 min and the hypertensive phase 3—5 min This was followed by permanent hypotension With these large doses, the pulse rate of the initial depressor phase differed clearly from that recorded during the later two phases For this reason the results were classified for analysis in the following way

I Pulse rate prior to infusion,

II Pulse rate in the initial depressor phase or within 1—2 minutes of the start of infusion

III Pulse rate after the initial depressor phase, or within 3—10 min of the start of infusion

After the first 10 minutes the pulse rate seemed to undergo no essential change

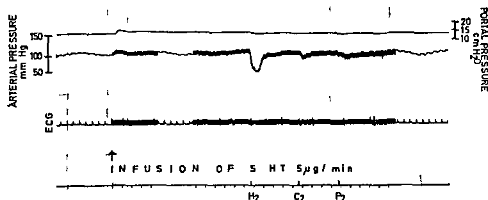


Fig 20 Endoportal 5 HT infusion 5 µg/min The graph shows the portal pressure arterial pressure and pulse rate as well as the ECG before and during the first 25 minutes of infusion Paper speed during the time the ECG pulse rate and amplitude were recorded was 5 cm/sec The first blood samples were drawn between the 10th and 20th minutes The unusually marked arterial pressure response associated with aspiration of the H sample was due to simultaneous closing of the inferior vena cava

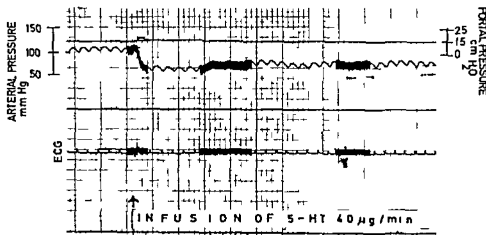


Fig 21 Endoportal 5 HT infusion 40 µg/min Effect on portal pressure arterial pressure pulse amplitude pulse rate and ECG Time in minutes below Paper speed during the recording of pulse and ECG was 5 cm/sec

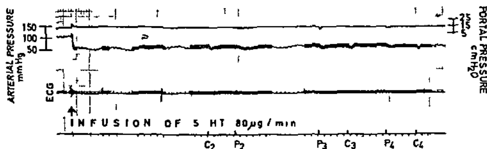


Fig 22 Endoportal 5 HT infusion 80 µg/min Effect on portal pressure arterial pressure pulse and ECG Time in minutes below Two samples were drawn between the 10th and 20th minutes two between the 20th and 30th minutes and two between the 30th and 40th minutes

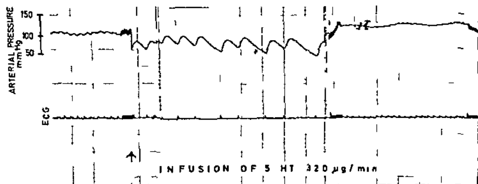


Fig 23 Endoportal 5 HT infusion 320 µg/min Effect on arterial pressure pulse amplitude pulse rate and ECG during the first few minutes of infusion Where the pulse wave is visible the paper advanced at a speed of 5 cm/sec

The results are presented in Table 11 and Figs 20—23 Distinct changes in pulse rates were not noted at endoportal doses of 5 HT below 80 µg/min Even then the pulse rates of the animals with monophasic arterial pressure response showed no change In animals with triphasic arterial pressure response bradycardia was almost always seen in the initial depressor phase and always subsequently tachycardia The pulse amplitude recorded from the carotid artery was lowest in the hypertensive phase The amplitude usually became more pronounced during the permanent hypotensive phase than it had been during the hypertensive phase

Endoportal infusions at the rates of 160 µg/min and 320 µg/min regularly produced bradycardia in the initial depressor phase, subsequently it always turned into definite tachycardia The rate of 320 µg/min

seen after vagotomy or atropinization (Page and McCubbin 1953, Schneider and Yonkman 1954, Salmoiraghi 1956)

The hypertensive phase following the initial depressor phase as was readily visible, was the most difficult for the animals It reflected perhaps most distinctly the direct pharmacological action of 5 HT on the circulatory system Heart action, in accordance with the chronotropic effect peculiar to 5 HT, was accelerated Besides this cardiogenic stimulation the increased peripheral resistance may also have contributed towards the hypertensive phase, as 5 HT at first caused vaso constriction

The hypertensive phase, seen at the beginning of the present experiments can therefore be explained on the basis of cardiac stimulation and vasoconstriction in other words, on the direct pharma cological effects of 5 HT On transition from the hypertensive phase to the permanent hypotensive phase the 5 HT contents of the blood did not fall but rather increased The rats condition however perceptibly improved The tachycardia remained, but the pulse amplitude improved Despite these findings, there was definite arterial hypotension compared with the initial pressure When serotonin has been infused directly into the extraportal vein of man or animal a tachycardiac and hypotensive response has also been obtained (Page 1958 Bojs 1961) Furthermore in addition to hypotension, the experiments revealed increased cardiac output and peripheral blood flow Maybe the tachycardiac hypotension which in the present experiments followed after the hypertensive phase, was also attributable to reduction of peripheral resistance

The dilatation of peripheral vessels can hardly be attributed to the direct action of 5 HT or to tachyphylaxis against it It is possible that the organism overcompensated for the vasoconstrictive effect of 5 HT Although 5 HT is known to release vasodilators (Feldberg and Smith 1953, Moore et al 1963) and although the metabolites of 5 HT may be vasodilative it is unknown whether the organism only uses humoral compensation mechanisms to eliminate the disturbance produced by excessice free 5 HT in cardiovascular functions In addition to the humoral mechanisms there are at least a nervous and a renal regulation system for the maintenance of normal vasomotorics (Guyton 1966)

Animal experiments, in which 5 HT has been infused or injected directly into the extraportal vein have revealed that arterial pressure response depends on the animal species used (Page and McCubbin 1953 Schneider and Yonkman 1954) on the size of the dose (Outschoorn and Jacob 1960) and site of administration (Erspamer 1961) The present

results do not contradict these findings They add however a new observation the capacity of the thrombocytes and liver to inactivate 5 HT, compared with the dose administered, determines the arterial pressure and pulse response associated with the endoportal 5 HT infusion When 5 HT is administered directly into the extraportal vein its action apparently is mainly correlated with the capacity of the thrombocytes to absorb 5 HT In this case only the lungs in addition to the thrombocytes can inhibit the access of free 5-HT into the area of systemic circulation

The platelets of the rabbit are able to absorb more 5 HT than those of the cat and those of the cat again more than those of the dog (Garattini and Valzelli 1965) Administration of 50 µg 5-HT per kg of body weight into the extraportal vein produces in the dog a pressor dominated triphasic arterial pressure response in the cat a depressor dominated triphasic response and in the rabbit a simple monophasic depressor response (Schneider and Yonkman 1954) It is therefore quite possible that the varying blood pressure effects recorded may be due primarily to the varying capacity of 5-HT inactivation of the thrombocytes of these animals Salmoiraghi et al (1956) found that on consecutive 5 HT injections of a given dose into the extraportal vein the triphasic arterial pressure response of rats became increasingly pressor dominated dose by dose Apparently the thrombocyte ability of inactivation diminished dose by dose as their degree of saturation with 5-HT increased

The skin flush present in the human carcinoid syndrome is a result of the dilatation of the small dermal vessels and capillaries In mild forms of the carcinoid syndrome the flush is different in character from advanced, severe cases (Thorson 1958 Snow 1963) Often the flush an early symptom is a transient flare of bright red lasting a few minutes It has usually not been associated with any other haemodynamic disorders (Snow 1963) but e g Sjoerdsma et al (1956) when they recorded the blood pressure during flushes of a few minutes noted a transient slight blood pressure fall The flush in severe carcinoid syndrome differs from the mild flush described above most markedly in that it persists for hours and even for days and is associated with severe tachycardia and a shock like hypotensive condition (Thorson 1958 Peart and Robertson 1961, Snow 1963)

In the present rat experiments it was found that an endoportal infusion of 5 HT at rates below 80 µg/min usually produced a relatively slight fall in arterial pressure (Figs 9 12 21) without

pulse or ECG changes This slight haemodynamic response seems to correspond well with the symptoms of carcinoid patients in early phases of the syndrome The haemodynamic effects produced by large 5 HT doses in rats, with tachycardia and definite hypotension, in their turn correspond to the vasomotor disorders seen in severe carcinoid syndrome

It is known that the mild symptoms produced by carcinoid do not always involve hyperserotoninaemia above the normal values (Sjoerdsma et al 1956 Pernow and Waldenstrom 1957, Peart and Robertson 1961) When the rats exhibited no pulse changes their extraportal 5-HT increased only by 50 100 per cent maximum 200 per cent Since the normal 5 HT contents of human blood are generally estimated to be 0 1—0 3 μg/ml (Gowenlock and Platt 1963) this means that a 200 per cent increase without control value may still fall within the range of values considered as normal It is therefore possible that a transient flush, associated with a carcinoid or dumping syndrome may indirectly result from increased liberation of 5 HT in the portal vein region although hyperserotoninaemia beyond normal limits may not be demonstrable

V GENERAL DISCUSSION

The rat was chosen to be the experimental animal for the study of the problems posed since it was inexpensive and had earlier been used in many studies analysing the metabolism and the haemodynamic effects of 5 HT (Salmoiraghi et al 1956 Dalgliesh and Dutton 1957 Keglevic et al 1959 McIsaac and Page 1959, Chadwick and Wilkinson 1960 Outschoorn and Jacob 1960, Airaksinen 1963 Tammisto 1965) An abundance of comparable data was therefore available Furthermore the anatomic structure of rat liver permits easy resection of parts of the liver without jeopardizing the animal s haemodynamics

The whole blood 5 HT content of the present rats 1 09 µg/ml was higher than the levels usually quoted in the literature This was partly due to the animal s large size (Bertaccini 1958) but apparently mainly to the fact that the Waalkes extraction and purification method was previously never used to determine the 5 HT of the rats whole blood The chemical method on the whole, gives higher levels than the biological method (Karki and Lahovaara 1965)

A method was developed for the perfusion of the rats intact liver by which while the vital functions (circulation and ventilation) were controlled the disappearance from the blood of 5-HT infused into the mesenterial vein during intrahepatic and extrahepatic circulation could be observed Since the total amount of blood in rats is small the method had to be one which did not require large quantities of blood for analyses 0 5 ml blood could be drawn from the experimental animals on 7 or 8 occasions without permanent haemodynamic or acid base disturbances provided care was taken to replace the quantity of blood removed by a physiological saline infusion into the mesenterial vein

The control experiments revealed that aspirations of blood and the endoportal isotonic physiological saline infusion (0 2 ml/min) did not appreciably affect the 5 HT contents of the blood The significant porta hepatic gradient of endogenous 5 HT (0 11 μg/ml), however did not emerge so clearly during the physiological saline infusion

The degradation of the endogenous 5-HT in the liver, demonstrated in the present experiments in the rat and earlier in the dog (Toh 1954, Drabanas and McDonald 1963) apparently presupposes a dynamic equilibrium between the 5 HT molecules of the platelets and the plasma Despite the widespread contradictory opinion there are on the basis of in vitro experiments distinct indications that a dynamic and not a static equilibrium prevails between the 5-HT molecules of the thrombocytes and the plasma with emphasis strongly on the thrombocyte side (Born and Gillson 1959, Hughes and Brodie 1959)

As blood flows in the sinusoids of the liver the 5 HT molecules of the plasma apparently easily come into the sphere of influence of the liver cells since the permeability of these sinusoidal walls is extremely high From the sinusoids even the proteins pass over into the extravascular space almost as easily as fluids (Guyton 1966) When liver cells metabolize the 5 HT plasma fraction, the result is a deficit To maintain the dynamic cell/plasma ratio of 5 HT the thrombocytes apparently very soon compensate for the deficit in the plasma fraction

In the initial phase of endoportal 5 HT infusion irrespective of the dose the 5-HT contents always increased in the extraportal circulation as well As small an endoportal dose of 5 HT as 5 μg/min produced a significant increase (19 22 per cent) Therefore the liver always initially removed only some of the infused 5 HT Not until the infusion had continued for 10 minutes did the rat liver begin to remove 5-HT from the blood at a rate equal to that of administration As this new finding was applied to the studies it could be demonstrated, using endoportal 5 HT infusions at different rates, that the capacity of rat s liver to inactivate 5 HT was about 80 μg/min With doses in excess of 80 μg/min the 5 HT in extrahepatic circulation regularly decreased more heavily, the larger the dose Up to the rate of 320 μg/min of endoportal 5 HT infusion, the rats were able to stop the continuous rise in the 5-HT contents of the extraportal blood towards the final phase of the experiments This stabilization admittedly did not occur until the 5 HT contents were very high A perfusion test of 40 minutes was not enough for it to occur in all the animals

These phenomena can be explained since the platelets and possibly

the red cells in the early phases of 5 HT infusions, according to numerous in vitro studies rapidly absorb some of the 5 HT infused into the blood and so participate in the inactivation of free 5 HT This inactivation, based on absorption by blood elements, ceased to be visible after about 10 minutes Then the liver was almost totally responsible for the biological inactivation of 5 HT infused endoportally When the endoportal 5 HT dose exceeded the great inactivation capacity of the liver the rats obviously began to utilize their extrahepatic inactivation mechanisms as well

When the arterial pressure pulse rate pulse amplitude and ECG were observed during infusions at different rates it was found that as long as the 5 HT inactivation mechanisms of the platelets blood cells and the liver were adequate to maintain a low 5-HT plasma content in extraportal circulation no (at 5 μg/min) or only slight changes were demonstrable in the extraportal haemodynamic functions Among the slight changes only a monophasic often very slight fall in arterial pressure could be recorded (dose < 80 μg/min) Once the 5 HT inactivation capacities of the liver and the blood elements were surpassed — the limit was always indicated by the extrahepatic 5 HT inactivation mechanisms entering into action — the arterial pressure response obtained always differed from that outlined above and the pulse changes were significant Bearing in mind that serotonin is a vaso constrictive amine contracting the smooth muscles (Rapport et al 1948 Sjoerdsma 1959) and that it has a positive inotropic and crono tropic action on the heart (Schneider and Yonkman 1954), the extra portal haemodynamic changes now recorded seemed to indicate in compliance with the present studies on 5 HT degradate, the borderline where an endoportal infusion of 5 HT surpassed the inactivation capacity of the blood elements and the liver

After partial hepatectomy, high extraportal hyperserotoninaemia and definite cardiovascular effects manifested themselves with considerably lower 5 HT doses than on perfusion of an intact liver Similarly when 5 HT was infused directly into extraportal circulation half the doses required for the purpose on endoportal infusion produced both a pronounced systemic hyperserotoninaemia and pronounced pulse and arterial pressure changes These findings showed conclusively that the role of the liver in the biological inactivation of 5 HT is important

On the basis of the present experimental findings it may also be assumed that the haemodynamic reactions the different animal species display to extraportal 5 HT doses of equal size in view of the animal s

body weight are apparently due both to the sensitivity to 5 HT peculiar to each animal species and the differing ability of the animals thrombocytes and possibly also that of their red cells to inactivate the infused 5-HT Confirmation of this hypothesis naturally requires comparative studies on different animal species If it holds good it will help finding a simpler explanation for the 5-HT haemodynamic responses which vary a great deal from one animal to another and for which many different kinds of explanations are offered today

No explanation based on experimental results has been found to date for the clinical findings why is the tumour producing a manifest carcinoid syndrome usually either located entirely outside the splanchnic region or else has sent metastases from the intestine to the liver (Thorson 1958 Snow 1963) It was now found in the present animal experiments that after the thrombocytes and possibly also other blood cells were »saturated» the liver was capable in vivo of eliminating almost completely the active plasma fraction of 5 HT despite a constant, very high rate of endoportal 5 HT infusion (80 µg/min) If this result could be applied to man with a body weight at least 250 times that of the animals employed in the present study, the human liver should be able to remove serotonin of intestinal origin at a minimum rate of 20 mg/min

It has been concluded from the effect of the removal of tumour tissue on the patient s 5 HIAA secretion that the carcinoid produces 2—3 mg 5 HT per gram of tumour tissue per hour (Sjoerdsma et al 1956) An argentaffinoma weighing 200 g may therefore produce 600 mg 5 HT per hour i e 10 mg/min According to the present results and at a conservative estimate the human liver would be able to eliminate the free 5 HT secreted by an intestinal carcinoid weighing 200 g provided it is as efficient as the rat liver Apparently in those few cases (Larmi 1962, Snow 1963) in which carcinoid located in the splanchnic region alone has produced a complete manifest syndrome the endoportally secreted 5-HT has apparently exceeded the said inactivation capacity of the liver either as a result of the large size or the exceptional activity of the tumour

VI SUMMARY

The purpose of the present study was to investigate the ability of the liver to inactivate 5 hydroxytryptamine (5 HT) infused by the endoportal route Another target was to determine whether the organism also uses extrahepatic mechanisms, in addition to the liver, for the biological inactivation of endoportally infused 5-HT Furthermore efforts were made to study the correlation if any, between the amount of 5 HT entering the extraportal circulation and the haemodynamic responses to 5 HT which have been found to vary a great deal

(1) To solve the problems posed a method was developed for perfusion of rat liver in vivo The disappearance of 5 HT from the blood in both intrahepatic and extrahepatic circulation could be observed with the aid of this method

(2) Since the quantities of blood available were small it was necessary to modify considerably the Waalkes chemical method for determination of the 5 HT of whole blood The 5 HT contents of the rat s whole blood correlated well with the 5-HT contents of rat serum quoted in the literature With the method of determination used the recovery of 5 HT was ca 80 per cent

(3) The rat s blood during intrahepatic circulation lost a small portion (ca one tenth) of its endogenous 5 HT This transhepatic gradient, present without the organism being loaded with exogenous 5 HT was considered to presuppose a dynamic equilibrium between the 5-HT molecules of the platelets and those of the plasma instead of the static equilibrium generally assumed The findings concerning the inactivation of exogenous 5 HT could also be understood with the aid of the dynamic cell/pla ma relationship of 5 HT

(4) When 5 HT was infused into the animals during 40 minutes the inactivation by thrombocytes and possibly by red cells based

as it seems mainly on absorption of 5 HT by these elements, was clearly visible only during the first few minutes Once the 5 HT infusion had gone on for over 10 min, the share of blood elements in inactivation of exogenous 5 HT ceased to be visible Only after this did the liver begin to remove 5-HT from the blood at a rate equal to that of the endoportal administration and not until this phase could the capacity of the animals liver to inactivate 5 HT be measured With the animals used in the present experiments, it was ca 80 µg/min because when 5-HT was infused endoportally at rates exceeding 80 µg/min, the organism always also used its extrahepatic systems of 5 HT degradation to an extent which could de distinctly recorded Similarly, the plasma samples taken from extraportal circulation began to show large quantities of free 5-HT only after the rate of endoportal infusion exceeded 80 µg/min

In some animals, an endoportal 5-HT dose of 320 µg/min exceeded the capacity of the whole organism to inactivate 5-HT during the period of observation

(5) The inactivation capacity of the remaining part of the liver after one third, and especially after two thirds of the liver had been resected was usually less than 40 µg/min

(6) When 5-HT was infused directly into extraportal circulation the 5-HT contents recorded in extraportal circulation were considerably higher than those following a corresponding 5 HT dose on endoportal administration

(7) When arterial pressures and pulse were recorded during 5 HT infusions at different rates the haemodynamic changes produced were found to depend very distinctly on the capacity of the blood elements and liver to inactivate 5 HT As long as this capacity was not exceeded there was at the most a monophasic depressor arterial pressure response without pulse changes Once the ability of the platelets (and possibly also other blood cells) and liver to maintain a low 5 HT plasma fraction was surpassed there were regularly very remarkable pulse and arterial pressure changes

(8) Since the location of a carcinoid tumour and the manifestation of its symptoms are correlated, it has been assumed that the liver if required is capable of preventing the access of 5 HT into extraportal circulation The high 5 HT inactivation capacity of rat liver observed in the present experiments supports this theory

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Supplementum 312

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KAROLINSKA INSTITUTET
STOCKHOLM 60 SWEDEN

ONTOGENY OF INTERHEMISPHERIC FUNCTIONS

AN ELECTROPHYSIOLOGICAL STUDY IN PRE AND POSTNATAL SHEEP

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CONTENTS

INTRODUCTION

The functional role of the great cerebral commissures notably the corpus cal losum which constitutes the largest tract of the forebrain remained for a long time somewhat of a mystery As late as 1949 McCulloch (cited by Bremer 1956) could make the almost classical remark that the sole function of the corpus callosum seems to be to mediate convulsive activity from one hemisphere to the other Actually at that time no clear cut neurological symptoms had been shown in patients subsequent to transection of the corpus callosum per formed as a remedy for generalized fits (Akelaitis 1940)

The history of the functional organization of corpus callosum goes back to 1683 when the famous anatomist Willis (cited by Hamilton 1885 see also Willis 1663) presented the theory that the corpus callosum is a »decussatio» of the motor fibers Later on the court surgeon of Louis XV La Peyronie in 1709 and 1744 presented a treatise to the French Academy in which he claimed the corpus callosum to be the site of the soul During the 19th century a great number of studies were devoted to the anatomy ontogenesis phylogenesis, phy siology and pathology of the corpus callosum This older literature has been reviewed in monographs by Levy Valensi (1910) and Mingazzini (1922) Suf fice to mention that Foerg (1855) seemed to be one of the first to advance the idea that the corpus callosum brings about »bei den sinnlichen Perception das Zusammenwirken beider Hemispharen» a standpoint which is not remote from that held by modern physiologists Excellent reviews on the structure and function of the neocortical commissures have been published by Bremer (Bre mer *et al* 1956 1958 1966 a see also Mamo 1965)

During the present centuary investigations on the functions of the cerebral commissures have been conducted along two somewhat different lines Behav ioural studies of animals in which the corpus callosum was transected were initiated by Soviet scientists of the Pavlovian school (*e g* Bykov 1924 — for references see Bianki 1958) They demonstrated that in these animals con ditioned reflexes could not be transferred from one half of the body to the other and concluded that the commissures are involved in the relaying of higher nervous functions between the two cerebral hemispheres

In the beginning of the 1950 s the investigations by Myers and Sperry on

the »split brain» preparation were started (references see Mountcastle 1962, Ettlinger 1965) These experiments are too well known to be once more reviewed and in this context it should only be pointed out that there is now convincing evidence that »engrams» or memory traces are mediated from one hemisphere to the other primarily via the corpus callosum Subsequent to the split brain method in behavioural studies on interhemispheric functions, Bures and Buresova (1960) have recently introduced the use of spreading depression for producing reversible and functional hemidecortication

The results obtained from animal experimentation on split brain preparations in later years also have been found to be valid for man The sequelae of callos ectomies in neurological patients submitted to specific test situations have been analyzed in a large number of papers which have greatly contributed to the understanding of different neurological conditions (*e g* Sperry 1961, Ge schwind 1965 Gazzaniga *et al* 1965 1967 reviews, see Potthoff and Umbach 1966 Holmes 1966)

The use of electrophysiological methods constitutes another approach to the study of interhemispheric callosal functions From the beginning these investi gations were primarily concerned with the capability of the cerebral commis sures to transmit different kinds of induced cortical activity from one side to the other One of the first to demonstrate this phenomenon seems to have been Gozzano who in 1936 showed that strychnine locally applied on the motor cor tex in one hemisphere caused spike activity to appear also in the other The transference of activity was abolished by section of the corpus callosum The method of strychnine neuronography was later used for systematic explorations of cortico cortical commissural connections (McCulloch and Garol 1941 Garol 1942) The papers most commonly cited in this context are those of Curtis (1940 a b) who introduced the concept of transcallosal response (TCR) for cortical potentials evoked by electrical surface stimulation of homologous areas in the contralateral hemisphere The properties of the TCR as well as its relation to other types of evoked potentials in adult animals have later been the subject of a large number of investigations (*e g* Bremer 1952, Chang 1953 a b, Perl and Whitlock 1955 Peacock 1957 Asanuma and Okamoto 1959 Grafstein 1959 Purpura and Girardo 1959 Clare *et al* 1961, Latimer and Kennedy 1961 Ajmone Marsan and Morillo 1963)

The functional significance of the callosal system in relation to specific afferent input has been examined in particular by Bremer In a series of papers Bremer demonstrated that sensory messages may be projected to both hemi spheres via a thalamo cortico cortical circuit, *i e* that the specific afferent input is relayed to the contralateral cortex by way of the callosal system (see Bremer 1966 b) Furthermore the cortical excitability for specific evoked re

sponses seems to be partly dependent upon a callosal influx the effect of which is primarily facilitatory The notion of an »action tonic dynamogenique reciproque» on the cortex of the two hemispheres from the callosal system was originally made by Claes (1939) and the change of this tonic callosal activity along with different states of wakefulness and sleep has been demonstrated by Berlucchi (1965)

In an attempt to reconcile the experimental results obtained by electrophysiological methods with the findings in behavioural studies on split brain preparations Bremer (1959) has defined the callosal functions as being fourfold

1 to maintain a dynamic equilibrium between the two hemispheres by a continuous exchange of cortico cortical impulses,
2 to relay sensory messages from one side to the other
3 to make possible the duplication of engrams imprinted in the association cortices
4 to make possible the integration of the two hemispheres into a common participation in acts of perception and in motor decisions

In addition to the cerebral commissural system the existence of extra callosal neocortico cortical connections has been shown more recently by Rutledge and Kennedy (1960) and there is good evidence that many different brain stem structures are involved (Rutledge and Kennedy 1961) Evoked interhemispheric potentials mediated via extra callosal pathways have been called interhemispheric delayed responses (IDR), and have hitherto been demonstrated only in cat Tentatively the extra callosal interhemispheric system has been assumed to serve an integrative function in multisensory inflow to the association areas of the cortex (Rutledge 1963) The functional significance of the system is however still not shown by any experimental data

Much discussion has centered on the significance of callosal and extra callosal mechanism for the interhemispheric coordination as represented by the phenomenon of bilateral synchrony in the EEG The synchronous bilateral appearance of the normal brain waves in man was originally described by Adrian and Matthews (1934) and in recent years this question has been studied with special reference to subcortical »pacemaker» mechanisms (Garoutte and Aird 1958) With the introduction of new methods of analysis of the EEG (cross correlation) quantitative estimates of the degree of bilateral synchrony have been obtained (Brazier and Casby 1952 Brazier and Barlow 1956 Kamp *et al* 1965) The availability of general purpose digital computers has further facilitated this and other types of EEG analysis (*e g* Brazier 1960 Adey *et al* 1961 Kaiser and Petersen 1966) Correlation analysis for the study of spontaneous activity led off from the exposed cortex has however been used for experimental purposes in animals to a very little extent (Brazier 1963)

Studies specially devoted to bilateral synchrony of the normal EEG in animals are relatively sparse The first seems to have been that of Travis and Dorsey (1932) who in unanesthetized rats found the activity within the motor areas to display the highest degree of bilateral synchrony Callosal section did not markedly alter this condition and the authors concluded that »corpus callosum alone is not responsible for the binding together of the two motor fields to ensure their unified functioning» A similar standpoint seems to have been taken in a recent study on monkeys (Batini *et al* 1967) Contrary to this view Claes (1939) and Bremer and Stoupel (1957) in similar investigations performed on cats claimed that the corpus callosum is of fundamental importance for the maintenance of bilateral synchrony of the normal EEG (see also Berlucchi 1966) The functional significance of non callosal mechanisms has also been pointed out and Aird and Garoutte (1958) supported the concept of a midline »pacemaker» which presumably is located in the brain stem and whose functioning could fully account for the occurrence of bilateral synchrony This opinion has been based both on experimental and clinical observations (Ogden *et al* 1956 Aird and Garoutte 1960)

The question of whether the integrity of the cerebral commissures is necessary for an interhemispheric transference of pathological *i e* convulsive activity has also been the subject of a large number of both experimental and clinical investigations The results however have been contradictory depending in part upon the species used and the type of convulsive activity studied In the dog it was demonstrated that epileptic *seizures* could be generalized to the whole body in spite of the fact that the corpus callosum was sectioned (Spiegel and Falkiewicz 1926) However callosal section prevented a spread of convulsive *activity* when induced by unilateral electrical stimulation both in the dog (Loeb 1951) and in the monkey (Erickson 1940) whereas no effect could be observed in the cat (Hoefer and Pool 1943 Straw and Mitchell 1967) More clear cut effects of callosectomy have ben obtained when studying epileptiform activity originating from an irritative focus produced by *e g* aluminium oxide When studied in acute experiments the activity in these cases was found to be completely confined to the one hemisphere following transection of the corpus callosum (Kopeloff *et al* 1950 Morrell 1960)

The method of callosal section in order to prevent generalized convulsions in humans was introduced by v Wagenen and Herren (1940) The therapeutic value of such operations however was doubtful in many cases and nowadays this treatment is reserved for very special indications Such patients have been carefully examined and Hursh (1945) could demonstrate that the bilateral synchrony of petit mal epileptic discharges seemed to persist after the operation

When summarizing the results from studies on the spreading of convulsive activity by callosal or/and non callosal mechanisms it can be concluded that neither provides the sole means of transmission and that apparently alternative pathways between the hemispheres can be utilized, partly depending upon the character of such an activity (*cf* Morin and Goldring 1950)

Cases of congenital agenesis of the corpus callosum have been known for a long time and were first described by Reil (1812) In modern times EEG studies have been performed on several of these patients but unfortunately the results have been contradictory and have contributed very little to a further understanding of the role of the commissures for bilateral synchrony (review see Toglia and Lapayowker 1966)

The fact that epileptic discharges *e g* 3/sec spike and wave pattern and possibly also normal EEG activity may appear bilaterally synchronous in the callosectomized brain warrants non commissural structures capable of inter hemispheric coordination (*e g* Hasegawa and Aird 1963, Guerrero Figueroa *et al* 1964) The significance of the mesencephalic reticular formation in this context has been demonstrated with the finding that unilateral lesions whithin this region causes asynchrony of the EEG (Cordeau and Mancia 1958) and the involvement of thalamic structures as part of a subcortical »driving» system common for both hemispheres is indicated by other experiments (*e g* Ralston and Ajmone Marsan 1956 Milhorat *et al* 1966) An account of the significance of non callosal ... brain stem structures for the functional inter relationship between the hemispheres has recently been given by Bremer (1966 c)

It has been pointed out that the term bilateral synchrony may be used in the sense of a wave to wave relationship as well as of a simultaneous occurrence of more long lasting EEG events *e g* spindle bursts (Hasegawa and Aird 1963) There are reasons to assume that callosal and non callosal mechanisms account for these two types of bilateral synchrony in different proportions (*cf* Berlucchi 1966) Therefore any definite standpoint on the significance of the corpus callosum in this context seems hardly justified

Recent studies performed on the developing organism have constituted a new and fruitful approach within the field of neurophysiology Apart from purely descriptive purposes such investigations have been undertaken with the hope that the dynamics of growth may facilitate the analysis of the basic problem of structure function relationship Thus developmental studies concerned with different phenomena in the cerebral cortex have undoubtedly contributed to the understanding of the functioning of cortical mechanisms in the mature animal as well Considering the large number of investigations devoted to the ontogenesis of different cerebral functions it is amazing that hitherto

very little interest has been paid to the question of how the two cerebral hemispheres become functionally interrelated during development

With the aim of studying the relationship between the development of functional properties and the myelination of central nerve tracts Ulett *et al* (1944) found that in the rabbit transcallosal responses could generally be elicited from the 4th day after birth although myelin was not be seen in the corpus callosum until later A more systematic investigation of the functional maturation of the callosal system was reported by Grafstein (1963 1964 a) on newborn kittens It was shown that the transcallosal response was already present at birth and that the shape of the response was in essence the same as that found in the adult cat Special interest was directed to the change of latency of the TCR with age as related to the formation of myelin According to Grafstein there are indications of two sets of callosal fibers with different sequences of myelination A similar problem was recently taken up by Verley (1967 a) when discussing the functional significance of the changing relation between conduction time and conduction velocity in the callosal fibers as found in the developing postnatal rabbit Finally the functional development of the callosal system in terms of interhemispheric transference of spreading depres sion has been examined in the rabbit (de Neverlee and Laget 1967) No stu dies are available of the functioning in early life of extra callosal interhemi spheric connections as represented by *e g* the interhemispheric delayed re sponse

Interhemispheric coordination as manifested by bilateral synchrony of the spontaneous cortical activity has hardly been studied at all in the immature individual in spite of the fact that the EEG development in many different species has otherwise been extensively described (references see Bernhard and Meyerson 1968) Dreyfus Brisac and Caveness seem to be the only investigators who have taken any notice of the phenomenon of bilateral synchrony in the developing organism It was found that in the premature baby bilateral syn chrony was not manifest until 8—9 months conceptional age (Dreyfus Brisac 1962) and in the monkey the waking activity becomes bilaterally synchonous at 3 months after birth (Caveness 1962) It should be added however that in the monkey the activity concomitant with drowsiness or light sleep may display bilateral synchrony already at birth Prenatal stages of the development were not studied in this animal

In the present electrophysiological investigation on the functional ontogene sis of interhemispheric relations two aspects have received special emphasis on the basis of the above background (1) interhemispheric evoked responses and (2) bilateral synchrony of the normal spontaneous cortical activity The experiments were performed on sheep during both pre and postnatal life

Preliminary reports on this investigation have been given (Meyerson 1966, 1967 a, b) The data processing system has been briefly reported on (Meyerson and Moller 1967)

ON THE MORPHOLOGY OF THE CORPUS CALLOSUM AND THE NEOCORTICAL COMMISSURAL SYSTEM

1 Phylogeny

It has long been known that a callosal commissural system appears in the phylogeny along with the evolution of the neocortex Thus a definite corpus callosum is only present in mammals with the exception of marsupials and monotremes in which the anterior commissure is exceptionally well developed instead (first observed by Owen 1837 — cited by Koelliker 1896) Electro physiological evidence for cortico cortical interhemispheric connections via the anterior commissure in marsupials has recently been obtained in the opossum (Nelson and Lende 1965) In higher mammals fibers originating in the neo cortex are found sparsely in the anterior commissure for example only small areas in the temporal lobe are interconnected by this commissure in the monkey (McCulloch and Garol 1941)

The first appearance in the phylogenetic series of a callosal commissural system is found in chiropterans and edentates where a rudimentary corpus callosum is present above and in front of the dominating anterior commissure (Smith 1899) The next step in evolution is represented by the rodents in which the corpus callosum has extended considerably in the posterior direction whereas the anterior commissure is less well developed In this order how ever, the anterior and posterior parts of the corpus callosum do not exhibit the characteristics of a *genu* and a *splenium* which are found in higher animals (Abbie 1939) The presence of these structures is said to be dependent upon the development of the frontal and occipital lobes found in higher animals (Auroux 1964 a *cf* v Mihalkovics 1877) Thus it was shown by Sunderland (1940) that in the monkey a proportionately large number of commissural fibers arise in the frontal areas In carnivores and ungulates the neocortical commissural system of the corpus callosum has reached an evolutional stage that is essentially comparable to that in primates and man (Bremer *et al* 1956 see also Świecimska 1967) According to the work by Schellenberg (1900) corpus callosum is relatively well developed in the sheep although slightly less than that in the goat It should however be noted that *genu* and *splenium* are definitely present in the sheep (*cf* Flower 1865) The similarity of the external configuration of the corpus callosum in a monkey (*Macaca irus*)

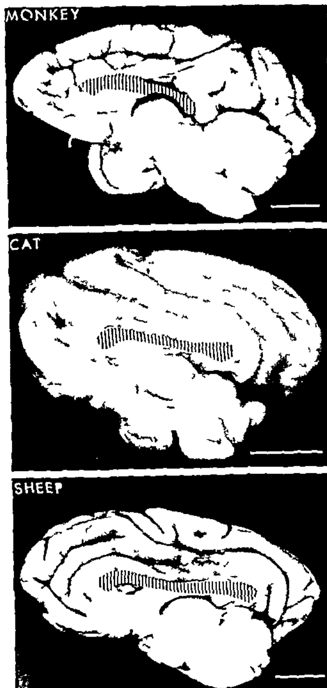


Fig 1 Sagittal mid ections of brains from adult monkey cat and sheep reproduced with equal sizes to show the relative extent of the corpus callosum Horizontal bar 1 cm

a cat and a sheep is illustrated in Fig 1 The hemispheres have been reproduced with the same anterior posterior size in order to facilitate comparisons of the relative extension of the corpus callosum in the different species

2 Ontogeny

In earlier neuroanatomical and neurophysiological literature a large number of investigations are found on the ontogeny of the cerebral commissures (references see Mingazzini 1922, Bremer *et al* 1956) Recently the same subject has been revised in morphological studies by Auroux (1964 a b) and Luttenberg (1964 1965 1966)

From observations in embryology pathology and teratology it is known that in all mammals the different cerebral commissures develop in the order of their phylogenetic ages Thus *e g* in the fetal rat (Auroux 1964 b) both the anterior and hippocampal commissures are formed during the 15—16th day whereas commissural fibers are not visible in the anlage of corpus callosum until four days later The comparatively late appearance of the corpus callosum is characteristic also for several other mammalian species (see Table 1)

The ontogeny of the corpus callosum in sheep embryos was studied by Osborn (1887) who found the first primordium to be present at about 44 days (145 full term) whereas Neumayer (1899) claimed that a rudimentary corpus callosum could be seen about ten days earlier These data should be compared with those found in other ungulates thus in pigs the corpus callosum is reported to be present at about 50 days (112 full term) (Tilney 1938) and in cows at 41 days (284 full term) (Patay *et al* 1959) Although the early embryonic development of corpus callosum is not the primary concern of the present study it was observed that a callosal commissure could definitely be demonstrated in sheep fetuses from the age of 46 days

In the early literature the role of *lamina terminalis* in the initial formation of the callosal commissure has been a matter of dispute Some authors held that this structure actually is the site of origin of the callosal fibers whereas others denied its importance in this respect (references see Auroux 1964 b) According to the present view *lamina terminalis* at least takes part in the formation of spongioblastic tissue which bridges the interhemispheric fissure and which constitutes the substrate for the ingrowing callosal fibers (Zuckerkandel 1909) According to Auroux (1964 a, b) the appearance of a corpus callosum in the midline is preceded by the formation of the so called callosal wings (»ailes du corps calleux») This term denotes the bulk of commissural fibers which are situated bilaterally between the cortex and the outer wall of the lateral ventricles The presence of callosal wings is first observed in the lateral part of the cerebral mantle and with advancing age they extend in

Table I
Appearance of corpus callosum in ontogeny

	crown rump length	fetal age	full term
Rat			
Zuckerkandel 1909	10 mm	18 d	22 d
Auroux 1964 a b	—	20 d	
Rabbit			
v Mihalkovics 1877	35—40 mm	16 d	32 d
Koelliker 1896	—	18 d	
Cat			
Martin 1895	40 mm	31—35 d	62 d
Cow			
Patay *et al* 1959	48 mm	45 d	284 d
Pig			
Blumenau 1891	80 mm	50 d	112 d
Dorello 1901 (cited by Mingazzini 1922)	80 mm	50 d	
Tilney 1938	83 mm	50 d	
Sheep			
Meckel 1815	40 mm	40 d	145 d
Kollman 1861	28—38 mm	35—40 d	
Osborn 1887	20 mm	32 d	
Neumayer 1899	49 mm	44 d	
Homo			
Levy Valensi 1910	—	4 mo	267 d
Hewitt 1962	84 mm	3½ mo	
Luttenberg 1964	70 mm	3 mo	

dorsal and medial direction in order to reach the interhemispheric fissure The concept of the bilateral centripetal origin of the corpus callosum is important for the interpretation of the development of the regional distribution of the transcallosal responses which will be dealt with in the present investigation

The principles of growth governing the subsequent phases of callosal morphogenesis have also been extensively discussed in the literature This problem

has recently been taken up by Luttenberg (1965) who states that corpus callosum is formed *in toto* i e the first primordium represents the whole later commissure and new fibers grow interstitially between those already existing (references see this author) This principle is denoted as intussusception in contrast to that of apposition which implies that only a part is present from the beginning and that new fibers are added to the first anlage one behind the other This latter opinion seems to be adopted by *e g* Auroux (1964 a) when he states that the frontal areas are the first to establish cortico cortical commissural connections In order to reconcile the two views de Villaverde (1919) assumed that the earliest phase of development proceeds by apposition until the main configuration is attained whereas a further increase in volume is caused by intussusception When examined in sagittal brain sections the macroscopic morphogenesis of corpus callosum in sheep embryos seems to fit into such a development scheme Thus in a 60 day fetus the configuration of the commissure has little resemblance to that in the mature animal whereas only about 8 days later a delineation of the future *genu truncus* and *splenium* can be easily recognized (Fig 2) The notion of an appositional formation of the corpus callosum in the early phase of its development is supported by the observation made on man (Bruce 1890) that malformations of the commissures dating from the last part of the 4th prenatal month generally imply partial agenesis of the corpus callosum, leaving the *genu* intact (*cf* Levy Valensi 1910 Bunts and Chaffee 1944, Auroux 1964 b)

3 Regional cortical distribution of callosal fibers

The extent and distribution of commissural connections in neocortex differ considerably among various species and Levy Valensi (1910) found that section of corpus callosum in monkeys caused more extensive cortical degenerations than in dogs Such species differences have also been pointed out recently (Myers 1965)

It has been generally assumed that the association areas of the cortex are more abundantly supplied with commissural connections than those representing motor and primary sensor projections (*e g* Chang 1953 a) This notion implies that there is in the neocortex an inverse relation between the number of callosal and thalamic fibers (Locke and Kruper 1965) Such a conclusion should not conceal the fact that the existence of interhemispheric connections between both motor and sensory areas have been demonstrated Thus in most species degeneration can be seen in area 4 and sometimes also in area 6 (Brodman) by producing lesions in corpus callosum or the contralateral cortex dog (Bianchi and d Abundo 1886—cited by Levy Valensi 1910 Muratoff 1893) rabbit (v Valkenburg 1913 de Villaverde 1924) rat (references see Jacobson









Fig 2 Medial views of fetal sheep brains of different ages with sagittal histological sections of the corpus allosum (Alzheimer—Mann stain) Horizontal bars in histographs 1 mm

1965) In an extensive recent investigation by Ebner and Myers (1965) motor commissural connections were found also in cat raccoon and monkey (see also Ebner and Myers 1962) although it was pointed out that these fibers were very thin and terminated in the deeper cortical layers where they seemed to form a dense net of ramifications

Electrophysiological methods for the exploration of commissural connections of the cortex were introduced by Curtis (1940 a) who studied the distribution of transcallosal responses in cats and monkeys According to Curtis such responses had the lowest threshold and the highest amplitude when evoked in the anterior suprasylvian gyrus (an association area) but could also be found in the motor areas It should be pointed out that responses were predominantly obtained from those regions of area 4 that represent the face the neck and the trunk whereas the hand and foot areas seemed to be poorly supplied by callosal fibers These findings were confirmed by McCulloch and Garol (1941) (monkey) Bailey *et al* (1941) (chimpanzee) and Garol (1942) (cat) using the method of strychnine neuronography Chang (1953 a) in a study of transcallosal responses concluded that the motor areas were better supplied by callosal fibers in the rabbit than in the cat Evidence for the functional significance of the transcallosal connections between the motor areas in cat has also been presented (Asanuma and Okamoto 1959, Purpura and Girado 1959)

Similarly to the distribution of callosal fibers within the motor area Ebner and Myers (1965) in both cat and raccoon found that in the primary sensory area (I) large callosal fibers were predominantly present in the trunk and face area The regions representing the distal parts of the extremities were poorly supplied (*cf* Bailey and v Bonin 1951) Furthermore, in the sensory area II no fibers could be observed which is in contrast to the study by Chang (1953 a) who in cat found this area to be particularly liable to produce transcallosal responses

The extent of the callosal connections between the visual areas has been the subject of a number of studies special reference to their possible importance for macular sparing (for discussion see Ettlinger 1965)

The extent and distribution of callosal cortico-cortical connections in ungulates are very sparsely referred to in the literature In connection with studies on the cortical motor representation in sheep King (1911 a) and Bagley (1922) incidentally noticed that lesion of the motor area caused degenerations both in the corpus callosum and in the contralateral cortex

4 Course of callosal fibers

The commissural arrangement of the callosal system, *i e* the interconnection

of the cortices of the two hemispheres, has long been recognized (Reil 1812 Foerg 1855) Following this discovery there was much discussion whether commissural fibers supply heterologous as well as homologous cortical areas (*e g* Sherrington 1889, Meynert 1892 Cajal 1955) The presence of commissural connections also to heterologous cortical areas has later been fully confirmed both with morphological techniques (*e g* Mettler 1936) and by the use of evoked potentials although such an organization of callosal fibers seems to be more elaborated in primates than in carnivores (Curtis 1940 a) The functional significance of heterologous interconnections has been demonstrated in man (*e g* Potthof and Umbach 1966)

It has lately been shown that in addition to the commissural fibers the corpus callosum also contains so called decussating pathways Locke and Yakovlev (1965) have demonstrated such a system by which *cingulum* and the contralateral *corpus striatum* is transcallosally interconnected by decussating fibers with oblique anterior posterior courses This organization has been found in several species

The existence of decussating fibers in corpus callosum directed to the contralateral *capsula interna* and to the pyramidal tracts has recently been assumed by Auroux (1964 a *cf* Mingazzini 1926) Such a non commissural callosal system has actually been reported from studies using refined techniques for the demonstration of degenerating fibers (Walberg and Brodal 1953) as well as from an electrophysiological investigation by Asanuma and Okuda (1962) It is of interest in this connection to note that Willis (1683) regarded corpus callosum as a structure in which corticofugal fibers decussated on their way to lower centra A similar view though largely modified, was taken by Hamilton (1885) who thought that the callosal fibers primarily serve as a connection between the cortex and *capsula interna* forming the »crossed callosal tracts»

5 Intracortical organization of callosal neurons

Descriptions of the intracortical organization of the commissural system are primarily based on results obtained with different degeneration techniques Correlative information has also been obtained with the method of laminar recording of evoked potentials A full account on the cytoarchitectonic features of the callosal neurons within the cortex comprises (1) the cells of origin of callosal fibers and (2) their terminals in the contralateral hemisphere

1 In the older anatomical literature most investigators agreed that the cells located in layer V and VI are the origin of callosal fibers (*e g* Muratoff 1893 Cajal 1955 v Valkenburg 1913 de Villaverde 1924) In connection with a functional investigation on transcallosal connections Chang (1953 a) also

claimed that the commissural neurons are mostly found in layer VI According to Chang the giant pyramidal cells probably do not contribute to the callosal system (*cf* Pines and Maiman 1939) On the other hand, Levy Valensi (1910) found that retrograde degeneration after callosal transection was most conspicuous of these cells whereas the small and medium sized cells were unaffected Auroux (1964 a) in his investigation on the development of the callosal system found that the fibers constituting the so called callosal wings appeared simultaneously with a differentiation of the fifth cortical layer

Recently the cortical location and organization of callosal neurons have been revised by Jacobson (1965) who argues that no cortical layer in particular gives rise to callosal fibers but instead that callosal cells can be found throughout the cortex (*cf* Grafstein 1959) Jacobson furthermore confirmed an observation by Lorente de No (1922) that axons of the large pyramidal cells bifurcate to corpus callosum and to striatum and thalamus

2 The terminals of the callosal afferents generally have little arborization in comparison with the specific thalamo cortical fibers (Cajal 1955) Chang (1953 a) in Golgi Cox preparations of intact brain found that callosal terminals were predominantly located in the superficial cortical layers, whereas Ebner and Myers (1965) claimed that few fibers were seen above the level of the third layer In the above mentioned study by Jacobson (1965) degeneration of callosal fibers was found to occur in all cortical layers with the exception of the most superficial (I) (see also Nauta 1954) Similarly Latimer and Kennedy (1961) when studying cortical unit activity concluded that the callosal terminals are distributed in the cortical layer II—VI Quite recently Jacobson (1966) in electron micrographs demonstrated that callosal terminals were most profusely found in layer V and VI The presence of numerous collaterals emerging from callosal fibers at the level of the deeper cortical layer was assumed by de Villaverde (1931 a b) and later confirmed by Ebner and Myers (1965) although the latter authors found considerable variations between different areas Chang (1953 a) assumed that the callosal terminals do not have any direct synaptic contact with the somata of the large pyramidal cells but with the distal portions of the apical dendrites (*cf* Nauta 1954 Globus and Scheibel 1967)

In a study on the early morphological development of isocortex in the sheep Åstrom (1967), using Golgi preparations, was able to demonstrate fibers presumably of callosal origin already in a 42 day fetus These fibers were situated in the outermost part of the matrix Not until about 58 days of age could afferent fibers (callosal or/and thalamic) be seen to penetrate into the cortex

METHODS

1 Animal material

The results on interhemispheric responses were based on experiments performed on 59 sheep fetuses out of wich 6 were twins 13 lambs and 6 adults Fetal ages ranged between 67 days and full term (about 145 days) and the lambs between 1 day and 3 months The development of bilateral synchrony of spontaneous cortical activity was investigated in acute experiments performed on 20 sheep fetuses of ages from 60 days to full term 4 lambs and 3 adults Most animals were of Gotland breed but some of Swedish landrace were also included in the material Young animals of these breeds generally weigh only about 30 kg In most case the day of impregnation was known and fetuses of appropriate ages could thus be chosen When unknown the gestational age was estimated using the tables relating age and weight published by Joubert (1956) Although that table has been constructed on data from different English breeds it closely resembles the age weight relationship of the Swedish ones used In the diagram in Fig 3 this is illustrated for the animals of known gestational ages from the present series (dots) which should be compared with the data of Joubert (solid lines) The unfilled circles represent values for twins the occurrence of which in these breeds are comparatively rare

2 Experimental procedure

During about 30 hours before the actual experiment the ewe was starved with free access only to water In order to avoid any possible protracted influence on the fetus premedication was not used The animal was tied to the operation table and under local anaesthetics a vein on the medial side of the foreleg was dissected free and cannulated A light dose of a short acting thiobarbiturate Thiogenal® (Merck Darmstadt) 25—30 mg/kg was slowly injected The animal was then tracheotomized and artificially ventilated since the barbiturate often seriously interfered with the spontaneous breathing The respirator was set to a minute volume of about 5 l A small amount of oxygen was regularly administered through the respirator Since the ewes had a marked tendency to develop bronchial spasm with artificial respiration adrenalin (0 4 mg s c) was given prophylactically

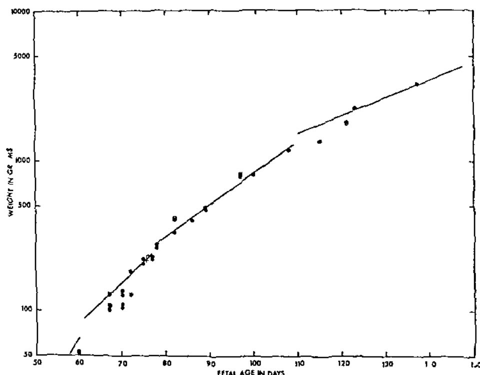


Fig 3 Age versus weight for fetal sheep according to Joubert (1956) (solid lines) compared with data from the present investigation (dots single fetuses circles twins)

The common carotid arteries were ligated and on one side a catheter was inserted in the proximal direction and was connected to a transducer for monitoring of the arterial blood pressure by continuous recording on a Grass polygraph With an anal thermistor the temperature of the ewe was controlled Ventriculostomy was routinely performed and the content was sucked out since in ruminants under general narcosis the bowel easily becomes distended leading to interference with the respiration

In order to avoid the occurrence of pulmonary atelectasis when lying on its side the animal was hoisted to an upright position by the aid of a hook inserted through the spinal ligaments and connected with a rope to a pulley in the ceiling The head was fixed to a headholder

Not until this stage was the animal permanently immobilized by the use of Flaxedil® (May and Baker) 1—2 mg/kg i v It should be mentioned that in ungulates gallamine has much less side effects than other muscle blocking drugs such as curare and succinyl choline It has recently been demonstrated

in cat that gallamine may cause a slight increase of the cortical excitability (Halpern and Black 1967) This effect was, however, attained with much larger doses, 6 mg/kg, than was used in the present investigation There was no indication of any placental transfer of the drug to the fetus

A wide unilateral craniotomy was then performed and the ewe was decerebrated with a section placed in the level of the posterior thalamus The skull cavity was emptied and great care was taken to control all possible sources of bleeding Until this stage of the experiment the animal had received a total amount of about 60 mg/kg of Thiogenal and thereafter no more anaesthetics was given Flaxedil had to be administered intermittently in low doses throughout the experiment As a routine a slow drip of Macrodex, 500 ml, was given

The ewe was then left to recover for about 2 hours Access to the abdomen was attained by twisting the hind body of the animal A caesarian section was made through a midline abdominal incision When opening the uterus care was taken to avoid damaging of the cotyledons Fetuses below 80 days of age could often be brought out through the incised uterine wall with the fetal membranes intact by gentle pressure of the uterus from behind In these cases the fetus could be seen floating in the transparent amniotic sac and its motility could conveniently be studied The fetus was then placed on a plastic cushion connected to a closed system of running water the temperature of which was regulated by a thermostat (Lauda) A heating lamp was placed above the fetus and its body was covered with cotton soaked in warm mineral oil Two different thermistors one in the anus and one inserted superficially in the skin of the back were used for monitoring the temperature of the fetus which was kept at 39°C — the normal temperature of sheep

The fetus was placed as near to the uterus as possible in order not to stretch the umbilical cord which unless manipulated with utmost care might go into spasm For this reason the cord was covered only with thin and light sheats of soaked cotton and was frequently inspected

For continuous EKG recording of the fetus thin silver pins were stuck into the skin on both sides of the forebody and the activity was then led off either to the polygraph or on an EEG machine (Grass) The fetal heart rate was generally 180—210/min which is consistent with values given by *e g* Barcroft (1946)

The head of the fetus was placed in a specially constructed headholder which was adaptable to fetuses of different sizes Since the bone structures of the skull are very soft in the youngest fetuses the head of these fetuses could not be firmly fixed and the pressure points of the headholder had to have a relatively large area The head was furthermore wrapped in soaked cotton leaving only its dorsal aspect uncovered The youngest fetuses as well as those

near term exhibited a lively spontaneous motility (*cf* Barcroft and Barron 1942) and therefore often had to be immobilized by Flaxedil No significant effect on the experimental result of the use of gallamine could be observed In the oldest fetuses the skin of the head was infiltrated by local anaesthetic Xylocain® (Astra)

Under dissection miscroscope a wide bilateral craniotomy was then performed and the dura was reflected Except for the oldest fetuses the skin of the head was so fragile that it was not possible to make a pool in the ordinary way Instead the cortical surface was covered with thin polyethylene sheats which proved to be very effective in preserving the moisture of the surface The exposed cortex was furthermore very frequently flushed by warm mineral oil Photos of the brain with the electrodes in place were taken with a camera set connected to the dissection microscope

The recording and stimulating electrodes were gently placed on the exposed cortical surface in homologous positions which in the lissencephalic stage was judged to be the same as a symmetrical arrangement In the gyrencephalic brain homology was ensured by taking in consideration also the convolutional pattern

When experiments were performed on lambs or adult sheep essentially the same technique was used *i e* the animals were immobilized artificially respirated and the general narcosis was discontinued All wound margins as well as the pressure points of the headholder were carefully infiltrated with local anaesthetic

For the description of the surgical method used for section of the corpus callosum see p 43

3 Recording system (*interhemispheric responses*)

It should be noted that the actual recording of brain potentials did not start until about 3 hours after the last dose of general anaesthetics had been given

The exploring reference and grounding electrodes were all matched calomel half cells connected to the preparation by agar saline bridges in polyethylene tubes with a recording area of 0 5 mm The reference electrode was placed on saline moistened cotton in direct contact with the most posterior part of the skull In the youngest fetuses in which high amplification had to be used the EKG often interfered with the cortical recording The most effective way of obtaining activity free of these artefacts was to ground the fetus via a saline moistened cotton strip wrapped around the neck

The electrodes were connected to a Grass P 6 DC amplifier and a Tektronix 502 CRO DC recording with an upper cut-off frequency of 0 5 or 2 KHz was used in all experiments and the drift of the system was generally less than

50 μV per hour A Grass kymograph camera provided photographic recording

The output of the oscilloscope was connected to a two channel tape recorder (ReVox with FM adaptation) One channel was used for the recorded activity and one for spoken information and a trigger signal (for use in subsequent processing) which was generated by a Grass S 4 stimulator synchronized with the stimulator set delivering pulses for brain stimulation The upper cut off frequency of the FM recording system was about 0 65 kHz

After the actual experiment the tape was replayed and after low pass filtering the demodulated signals were fed into a Computer of Average Transients (CAT 400 A) Averaging of gross potentials is generally used only when a large number of responses are available for processing In the fetus, however the »fatiguability» of the central nervous system does generally not permit more than 10—20 consecutive responses to be obtained from one and the same cortical position before a considerable decrease in the excitability becomes apparent Since the »improvement» of a potential with averaging is proportional to the square root of the number of responses even the use of comparatively few responses for averaging may sometimes be meaningful The averaging system was furthermore found to be of value for reducing the stimulus artefact which with the use of high stimulation voltages often was inevitable particularly in the youngest fetuses Thus summating the same number of responses obtained with both polarities of stimulus could result in an averaged response with a very faint stimulation artefact

The sweep time of the CAT was usually set to 0 5 sec and the averaged signal was led to the Tektronix CRO for photographing

The electrodes for surface stimulation were paired silverwires ball tipped with a diameter of about 0 5 and an interspace of 1 mm When adapted for direct stimulation of the corpus callosum different kinds of concentric and parallel needle electrodes were used Stimulus pulses were delivered from one or two Grass stimulators S 4 with RF isolation units The duration of the stimulus pulse was set to 0 15 msec except for use in the youngest fetuses in which longer durations often had to be employed (1—5 msec) No attempt was made to control the stimulus current but for the matter of general orientation it can be mentioned that with this system, the range of stimulus voltage generally used 25—90 V, approximately corresponded to 0 5—1 5 mA

4 Recording system (*spontaneous electrocortical activity*)

For this part of the investigation the animals were prepared in exactly the same way as for the experiments in which interhemispheric responses were studied and actually both types of experiments were performed on some preparations The same recording electrodes were used

The activity was recorded on a 6 channel Grass EEG machine The ink writers were synchronized within 0 1 sec Recordings were exclusively made from the exposed cortical surface and a monopolar configuration with two exploring electrodes connected to the same reference electrode placed on the posterior part of the skull was generally used One additional channel of the EEG machine was often used to record activity *between* the two exploring electrodes since, in that way the spurious effect of events in the locus of the common reference electrode could be accounted for in the analysis of bilateral synchrony In addition, a large number of recording periods was performed with the use of other locations of the reference electrode(s) on the frontal sinus on the ears (interconnected or connected each to one exploring electrode) or on the remaining midline bone strip In a few cases control recordings with a bipolar arrangement on each hemisphere were performed

For automatic computer analyses of the activity, periods of 1 min each were recorded on a FM tape recorder connected to the amplifier stage next to the last of the EEG machine In several cases periods of 2 min were also used In the youngest fetuses below the age of about 80 days the spontaneous activity is discontinuous *i e* displaying only limited epochs of activity with long isopotential intervals In order to reduce the cost of data processing these intervals were cut out from the tape prior to the analogue to digital conversion The final tape recordings from these fetuses thus consisted of several short periods of activity generally 4—5 sampled from both channels simultaneously and put together to cover a time of 1 min In several cases up to four such one minute periods were obtained from one continuous original recording lasting for 5—10 min

Altogether about 650 one minute periods of activity were subjected to automatic analysis (auto correlation and spectral analyses for each of the two leads and their cross correlations) For further details on the recording system and the data processing see Appendix (p 110)

5 Calculation of maximal callosal conduction velocity

The onset and peak latencies as well as the durations of the different components of the interhemispheric response (IHR) have been measured In most cases CAT averaged responses were used Measurements were made for each of all the different subareas (see p 32) and since the IHR was always recorded from several points within each subarea in one and the same animal the average values were used

Polynomial approximation of the fourth order to the experimental data was performed on an IBM 7090 computer for each area and component

Examples of such curve functions are given together with the raw values in Fig 23—25 A

In order to estimate the conduction velocity the cortico cortical distance was measured on coronal sections of the formalin fixed brains The measurements were made with the aid of a thread laid directly on these sections or on photographs along the assumed callosal path On the basis of these measurements and the polynomial curves of the onset latency of the TCR, approximate values of maximal callosal conduction velocity were estimated

6 Histological technique

The histological investigation on the myelination of the corpus callosum in the sheep was based on the examination of more than 30 brains from fetuses and lambs After the electrophysiological experiment the brain was removed and immersed in 10 % formalin with 0 9 % saline and later sectioned in the coronal plane The sections were mounted in celloidin and 20 μ thick slices were prepared In most cases a slightly modified Loyez staining for myelin was used (original method, see *e g* Drury and Wallington 1967) since this method has been shown to give reliable results on developing nervous tissue (Marty 1962 Yakovlev and Lecours 1967)

Although great care was taken to standardize all steps in the staining procedure the intensity of staining could sometimes vary in slices obtained from different fetuses of the same age In order to evaluate these variations a section from one and the same brain of a lamb in which the myelination of the corpus callosum was half way terminated was stained together with each of the other brains In addition to the myelin staining several brains were also prepared using the methods of Holmes Landau or Alzheimer Mann in an attempt to examine the course and regional distribution of the callosal fibers These sections were mounted in paraffin

RESULTS

ON THE MORPHOLOGY OF THE ADULT SHEEP BRAIN

The ungulate brain displays a convolutional pattern that is relatively complicated in comparison with e.g. that of the carnivores. The gross anatomy of the dorsal surface of the brain of an adult sheep is shown in Fig. 4. The nomenclature of the convolutions and fissures varies in the literature and the terminology used in this study has been adopted from publications by Krueg (1878), King (1911 b), Bagley (1922), Landacre (1930) and Rose (1942) and more recently by Richard (1967).

Like in other ungulates the precentral region in the sheep is predominantly occupied by *g. frontalis superior* which in the carnivores is equivalent to *g. sig*

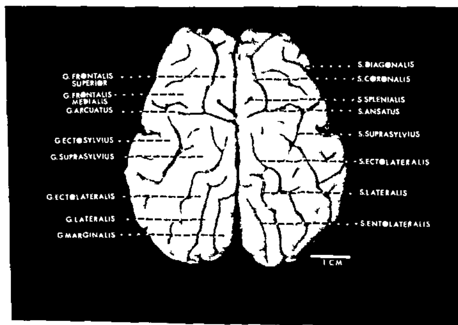


Fig. 4. Dorsal view of adult sheep brain (Gotland breed 24 kg) with identification of fissures and convolutions as used in the present investigation.

moides This gyrus is sharply delimited laterally by *s coronalis* and posteriorly by *s ansatus* the latter corresponding to *s cruciatus* of the carnivores In several studies on the ungulate brain *g frontalis superior* has been proven to be the main motor area, and morphologically it is characterized by the presence of giant pyramidal cells in the cortical layer V (Koppen and Loewenstein 1906 Simpson and King 1911 Bell and Lawn 1956 Breazile *et al* 1966 Barone *et al* 1966) There is some disagreement concerning the extension of the gigantopyramidal layer into the posterior part of this gyrus located between *s splenialis* and *s ansatus* (*cf* King 1911 b Rose 1942) Giant pyramidal cells as well as a cortical lamination characteristic for the motor area have been claimed to be typical also for the following areas (Bagley 1922) the medial region of *g frontalis medialis* the most anterior part of *g suprasylvius* and the small gyrus which joins *g suprasylvius* and *g frontalis medialis* and which has been given the name »Übergangswindlung» (Schellenberg 1900) or *g arcuatus* (Simpson and King 1911) However the motor functions of these areas are uncertain and the reliability of the histological findings have been questioned by Rose (1942)

The somato-sensory areas in the sheep have been investigated by Adrian (1943) and Woolsey and Fairman (1946) According to these authors the posterior region of *g frontalis medialis* together with a minor area within the anterior part of the *g ectosylvius* are sites of tactile sensory projection Like in other ungulates there is a marked dominance of the sensory representation for the nose and the lips Auditory and visual functions have not been studied in ungulates but there are reasons to locate the primary projections for these modalities to *g ectosylvius* and to *g marginalis* and *lateralis* respectively (Rose 1942) By analogy to the carnivores it is furthermore highly probable that *g suprasylvius* is to be classified as being primarily an association area

DEVELOPMENT OF FISSURES AND CONVOLUTIONS

The course of the gross anatomical development of the ungulate brain has been the subject of a few earlier studies Already in 1815 Meckel in his review on the ontogenesis of the central nervous system of mammals, included a description of the fetal sheep brain Later on Kreug (1878) gave a thorough description of the development of the sulci in the ungulate brain and special studies were devoted to the sheep brain by Anthony and de Grzybowski (1936) and by Barron (1950) The character and aim of the present investigation made it necessary to know more about the development of the convolutional pattern than what is available from these earlier studies The following description as well

as the series of drawings in Fig 5 are based on photos of 60 brains from fetuses and lambs of different ages The age was known by the date of settlement or birth (see Methods)

Up to the age of 70 days the brain of the fetal sheep is lissencephalic A few days later there develops on the dorsal surface a shallow and broad depression which denotes the site of the future *s suprasylvius* At this stage the brain measures about 2 cm at its largest transversal plane The total weight of the fetus is about 100 g and its crown rump length is 14 cm At about the 75th day the anlage of the *s coronalis* appears *S suprasylvius* becomes deeper and its rostral and caudal extremities are situated more laterally indicating its future angular appearance At about the 85th day it is possible to trace the convolutional pattern of the adult sheep At this age the *s coronalis* has developed into a definite fissure and its posterior part has made a turn medially, the future *s ansatus* Thus from now on the frontal part of the fetal brain is anatomically divided in a *g frontalis superior* (motor) and a *g frontalis medialis* (sensor) In the posterior part of the dorsal surface a short and shallow *s lateralis* appears half way between *s suprasylvius* and *fissura interhemispherica* At the 90th day a short sulcus in the middle of *g frontalis medialis* appears as well as *s splenialis* the rostral part of which appears medially in *g frontalis superior* Between *s lateralis* and *s suprasylvius* there is an indication of a new sulcus, *s ectolateralis* which extends further in frontal direction during the 95th to the 100th day Besides a short *s entolateralis* appears medially of *s lateralis* and thus all sulci of the adult brain are present Now *s splenialis* is deep and nearly divides *g frontalis superior* in a shorter posterior and an anterior more elongated part *S ansatus* extends to the medial border of the hemisphere and due to the development of its lateral extension *g arcuatus* can be relatively well delimited

Although not particularly pronounced in the sheep there are certain individual variations in the convolutional pattern (Rogner 1883) and from the age of 95—100 days these will eventually disguise the slight changes in the brain morphology that takes place during later stages of development

During the period between 110 days and birth the brain displays a convolutional pattern that does not essentially differ from that of the adult The general appearance of the dorsal surface gives an impression of increasing complexity in that the course of the sulci becomes more convoluted Besides they become deeper and there seems to be an increased »packing» of the gyri

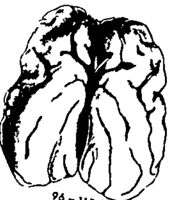












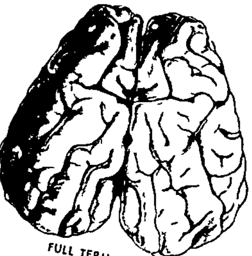


Fig 5 Development of fissures and convolutions in the fetal sheep Relative size of the brains is approximate

A INTERHEMISPHERIC RESPONSES IN THE ADULT SHEEP

In order to simplify the following description the *entire* sequence of potentials that can be recorded from the cortical surface in response to electrical stimulation of a homologous point of the contralateral hemisphere will be referred to as the *interhemispheric response* (IHR) This term thus includes both the so-called transcallosal response (TCR Curtis 1940 a) and the interhemispheric delayed response (IDR Rutledge and Kennedy 1960) as described in other species

1 Configuration

The mapping of IHR s in the intact adult sheep brain was performed with single monophasic stimuli of supramaximal voltage (0 1 msec duration) delivered at intervals of about ten seconds At least ten responses induced by stimulus pulses of each polarity were recorded and then averaged in the CAT apparatus The electrodes were placed on symmetrical areas as judged from the cortical topography and the location of the recording electrode was then adjusted to obtain a response with maximal amplitude

The general configuration of the IHR in the adult sheep is essentially similar to that described for other animals Thus in those areas which most readily give consistent responses in the sheep—*e g* the motor area— the main feature of the initial part of the IHR was a positive negative sequence of potentials, the negative phase generally being the most prominent (Fig 6 A) For the sake of simplicity the positive and negative phases will be referred to as P_1 and N_1 respectively The onset and peak latencies of the P_1 phase amounted to 6—8 and 10—12 msec respectively and the corresponding values for the N_1 component was 12—14 and 15—20 msec The duration of P_1 was about 10 msec and of N_1 20—25 msec

With the use of high stimulus voltage the initial response was followed by a long latency component whose configuration was more variable than the early response and may consist of either a deep and slow positive wave (P_x) immediately following the N_1 component (Fig 6 C) or of a long lasting high amplitude negative plateau (N_- Fig 6 D) The latency of the late component was variable the onset value generally being 30—70 msec In a few cases the late component consisted of a positive positive negative or even a triphasic negative positive negative deflection and appeared separated from the initial P_1—N_1 complex with an onset latency of more than 150 msec As will be shown (see page 43) the P_1—N_1 complex of the IHR corresponds to the TCR described in other animals while the late components correspond to the IDR

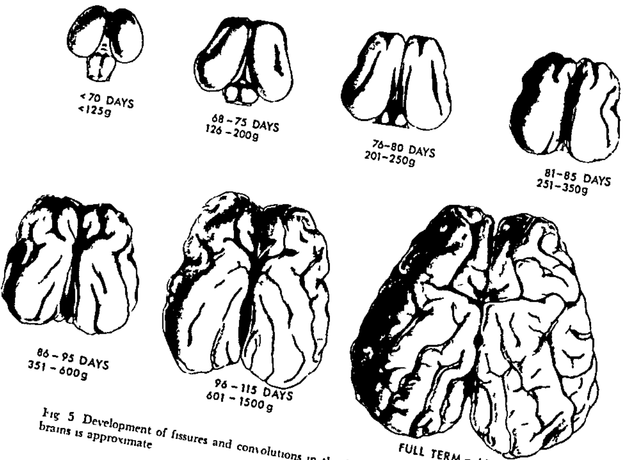


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frontalis superior In some cases however when recorded from the posterior lateral part of the gyrus the initial complex was found to be purely positive (see Fig 9, lamb 23 days)

G arcuatus

The small gyrus which surrounds the lateral extremity of *s ansatus* and which has been called *g arcuatus* (see above) deserves special attention since well defined interhemispheric responses were readily obtained in this area *G arcuatus* represents a frontal continuation of *g suprasylvius* and its functional relationship with this area is indicated by the fact that the configuration of its IHR sometimes resembled that obtained from *g suprasylvius*

G suprasylvius g lateralis and g entolateralis

In these gyri the threshold for the IHR was found to be high Within *g suprasylvius* responses were most easily obtained from the frontal and occipital portions whereas the middle part only occasionally produced identifiable responses In *g lateralis* the most consistent responses were obtained in its posterior portion The configuration of the IHR induced in these areas were variable The initial P_1—N_1 complex usually had a low amplitude and was often buried in the »noise» of spontaneous cortical activity The late component of the IHR obtained from these areas was generally of the P_2 type

G ectosylvius

From *g ectosylvius* responses were consistently obtained The responses recorded from its medial area adjacent to *s suprasylvius* were almost comparable to those obtained from the motor area Chang (1953 a) in his study of the TCR in cats found that the responses obtained from *g ectosylvius* generally display an inverted pattern *i e* the initial negative phase N_1 is replaced by a positivity Such positive monophasic responses have occasionally been observed in the sheep but were not at all confined to *g ectosylvius*

In *summary* interhemispheric responses in the adult sheep consist of an initial positive deflection followed by a negativity of higher amplitude and consistency Late components appearing after the early positive negative complex are occasionally obtained in response to high stimulus voltage The configuration of the early and late components resembles that of the transcallosal response and of the interhemispheric delayed response respectively in other adult animals Responses are most easily obtained from *g frontalis superior* the posterior parts of *g frontalis medialis* and the medial parts of *g ectosylvius*

Well defined IHR s are rarely found in the frontal and middle parts of *g suprasylvius*

DEVELOPMENT OF INTERHEMISPHERIC RESPONSES

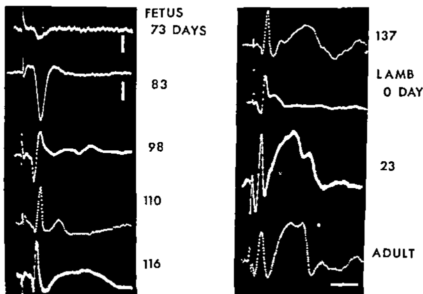
It was found that an IHR could not be evoked in fetuses younger than 67—68 days (weight about 100 g) Definite responses were obtained at this age and older in all fetuses except two (67 and 70 days)

1 Configuration

In the young fetuses the general configuration of the IHR was the same regardless of the region from which it was obtained Typical examples of re cords obtained from five different areas during different phases of develop ment are depicted in Fig 7—11 Supramaximal stimulation has been used throughout and the electrodes were placed symmetrically All but three (Fig 7 73 and 83 days Fig 8 73 days) of the responses are averaged During the first developmental phase, *70—85 days* the IHR was characterized by a dominance of the positive phase (P_1), which was occasionally followed by a negativity (N_1) of a comparatively long duration The amplitude of both com ponents was low (see below)

At the age of about *85 days* the negative phase (N_1) of the IHR appeared more consistently as to become the dominant component later in development At the same time the amplitude of P_1 successively diminished Since there was also a decrease of the total duration of the P_1—N_1 complex the general form of the response resembled more closely that of the mature animal The transition to the more mature form of IHR was first seen in *g frontalis superior* (Fig 7) *g arcuatus* (Fig 8) and in the posterior region of *g frontalis medialis* (Fig 9) At *95—100 days* this form of the response was regularly also found in the middle and frontal parts of *g frontalis medialis* and in *g ectosylvius* (Fig 10)

As pointed out above IHR s are difficult to obtain from *g suprasylvius* in the adult sheep Judged from the gross anatomical development the frontal and middle parts of this area in the adult presumably correspond to those from which responses first could be recorded in the fetus As seen in the first three records in Fig 11 the initial development of the IHR in *g suprasylvius* fol lowed the general trend described above Thus, the response was first dom inated by a positive phase and at an age of about 80 days the N_1 component appeared In contrast to the development of the IHR in other areas a prim itive pattern of the initial P_1—N_1 complex of the response recorded from *g*



Figs 7—11 Development of interhemispheric responses obtained from different areas with supramaximal stimulation of homologous points All but the first two responses in Fig 7 and the first response in Fig 9 are averaged Calibration in Fig 7 50 μV (first record) 100 μV (second record) Horizontal bar in Figs 7—11 100 msec

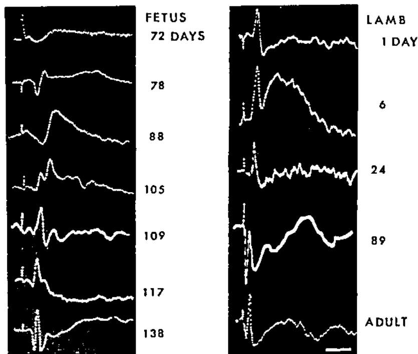


Fig 8 Legend see Fig 7

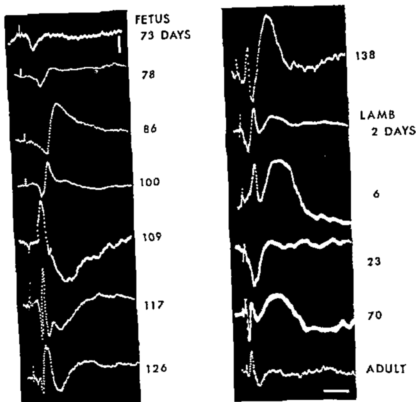


Fig 9 Legend see Fig 7 Calibration 50 µV (first record only)

suprasylvius remained unchanged even during later stages of development and the well synchronized high amplitude P_1—N_1 components were never seen in this area The records depicted in Fig 11 illustrate that even in the mature animal the pattern of the early components of the IHR was highly variable within *g suprasylvius*

It should be pointed out that in some records in Figs 7—11 a low amplitude negative deflection can be seen to appear with a very short latency This potential is probably part of a DCR induced by the spread of stimulating current across the midline (see above)

By the age of about *100 days* the positive negative complex was generally followed by late components These components appeared in *g suprasylvius* (Fig 11) earlier than in the other regions The configuration of this part of the response was highly variable and may appear either as a deep positivity following directly on the falling slope of the negativity as a long lasting negative

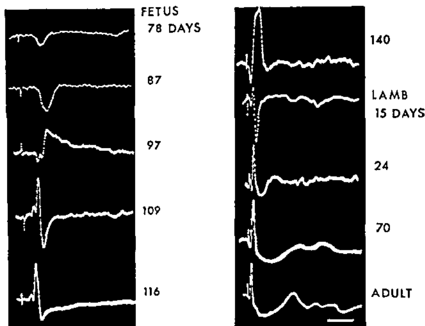


Fig 10 Legend see Fig 7

plateau or in some cases as a separate sequence of potentials usually positive negative and sometimes even triphasic As seen in Fig 7 the responses from *g frontalis superior* generally exhibited a late component in the form of a negative plateau whereas the form of those recorded from *g frontalis medialis* (Fig 9) and *g arcuatus* (Fig 8) was more variable In *g ectosylvius* (Fig 10) the late components were as a rule not as prominent as in the other areas

Late components appearing as a separate complex of potentials could only be obtained from *g suprasylvius* During a limited phase of development 90—110 days they were very prominent and in fact often dominated the response (see the sixth record in Fig 11) Later in development late components of this type appeared rarely and were replaced by more inconsistent positive deflections following upon the N_1 component It should be added that although *g arcuatus* seems to be topographically closely related to the frontal part of *g suprasylvius* the late components of the IHR induced within this area were occasionally similar to those found in *g frontalis superior*

The variations in amplitude and shape of the late component at the age of 90—110 days were sometimes observed to be related to the appearance of the spontaneous cortical activity At this developmental stage the EEG dis

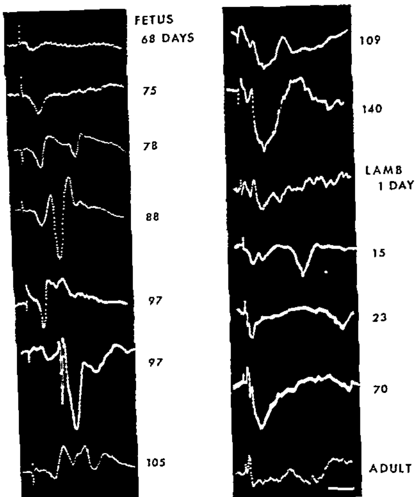


Fig 11 Legend see Fig 7

plays certain traits of immaturity in that so-called PN I bursts (Bernhard *et al* 1959) may still be present (see below) When an IHR was induced simultaneously with the appearence of such a PN I burst it usually exhibited a well developed late component

The presentation of the characteristics of the developing IHR has intentionally stressed the general properties of the configuration common to responses obtained from different parts within a particular area and from different individuals However this should not conceal the fact that, as in most types of gross potentials recorded from the cortical surface, there were sub

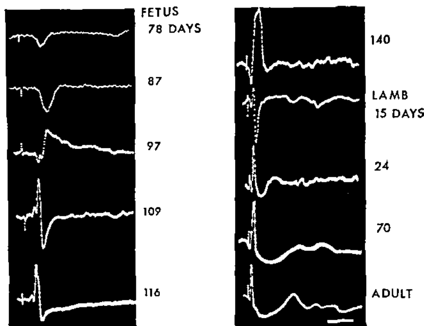


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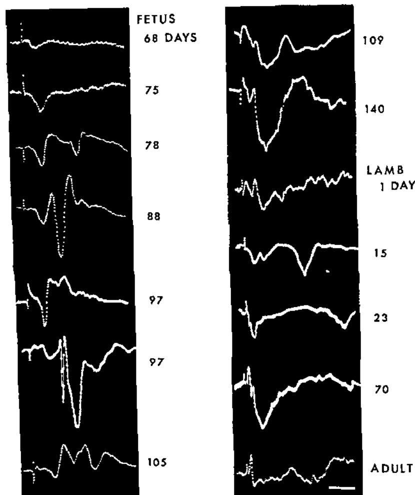


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stantial variations—intra as well as interindividual Besides, as illustrated in Fig 12, changes in the configuration of the response may occasionally occur even spontaneously without any change of the experimental conditions The responses reproduced in the figure were obtained from a 123 days old fetus with intervals of about 15 seconds and with the stimulus voltage kept constant This finding illustrates the advantage of using CAT averaged responses for the analysis



Fig 12 Three consecutive records of interhemispheric responses obtained from *g frontalis medialis* in a 123 day fetus Stimuli delivered at intervals of about 15 seconds Calibration 100 μV 100 msec

It should moreover be noted that although great care was taken to place the stimulating and recording electrodes symmetrically responses could sometimes be found to appear with an inverted pattern i.e. the N_1 component was replaced by a positive phase Considerable differences in the configuration could also be found when comparing responses evoked in nearby regions within one and the same area An example is given in Fig 13 showing IHR s obtained from four different points on *g ectosylvius* in a 100 days old fetus The electrodes were carefully placed in a symmetrical arrangement In this case the N_1 component can be identified in all records though it has a variable configuration A late component in the form of a slow negativity, is clarely present only in the uppermost left record

2 Threshold

When the IHR first appeared during development, the threshold was very high With a duration of 0 15 msec the threshold voltages was 40—70 V or more compared to about 10 V for that of lambs and adults At a gestational age of 80—85 days the threshold of the IHR when evoked within areas most liable to produce responses generally approached that of the newborn and adult animal

In the youngest fetuses (70—75 days) the shape of the responses was not affected by the stimulation strength once the threshold was attained Thus, in these fetuses the IHR always consisted of a low amplitude positive deflec

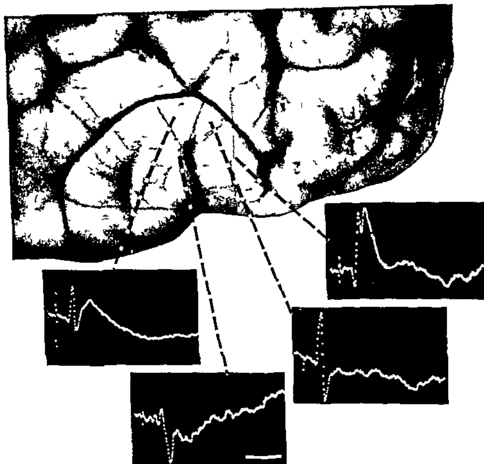


Fig 13 Interhemispheric responses recorded from different locations within *g ecto sylvius* of a 109 day fetus Supramaximal stimulus voltage in all records Averaged responses Horizontal bar 100 msec

tion occasionally followed by a negativity From the age of 85—90 days an increase of the stimulus voltage over the active cortex resulted in definite changes in the configuration of the response The effect of changing the stimulus voltage in three different experiments is shown in Fig 14 As a rule stimulation with threshold voltage induced a response consisting solely of the N_1 potential (Fig 14 A) Increasing the voltage by about 50 % caused the appearance of a preceeding P_1 component (Fig 14 B and E) which resulted in a reduction of the onset latency of the response In more mature fetuses and in postnatal animals the P_1 component was however not always present The only result of increasing the stimulus voltage was an augmentation of the amplitude of N_1 which is illustrated in Fig 14 G—I where the CAT averaged recordings

are all based on the same number of responses. The absence of a P_1 component is furthermore apparent in several records in Fig. 7—11 which were all obtained with supramaximal stimulation.

Late components of the IHR could only be obtained with medium or high stimulus voltage, the value of which sometimes had to be twice that of the threshold for the P_1—N_1 complex. Two entirely different types of late components in response to a high stimulus voltage are shown in Fig. 14 F and 14 H and I.

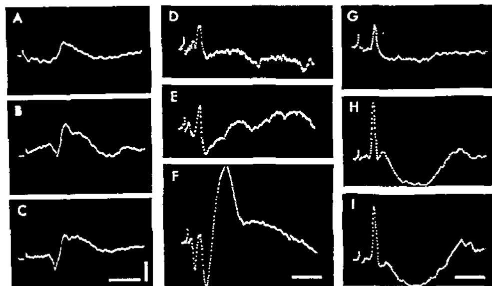


Fig. 14. Effect on interhemispheric responses of increasing stimulus voltage. A—C, records from a 111 day fetus (*g. frontalis medialis*) with 40 (threshold), 60 and 80 V. D—F, records from a 6 day lamb (*g. ectosylvius*) with 60, 90 and 125 V. G—J, records from a 126 day fetus (*g. frontalis superior*) with 20, 35 and 60 V. C, F and J, responses with supramaximal stimulation. D—I, averaged responses based on the same number of single responses. Calibration: 100 µV (A—C), 50 msec (A—C), 100 msec (D—J).

In *summary*, the IHR develops from a simple monophasic positive configuration to a diphasic positive-negative pattern in which the negative potential becomes dominating. The most consistent shape of the response is found in records from *g. frontalis superior*. Late components appear later in development than the initial positive-negative complex and have variable forms. During a certain phase of development the late components dominate the response in *g. suprasylvius*.

In the youngest fetuses the form of the IHR is unchanged when stimulus voltage is increased above threshold. Later in development the IHR in re

sponse to threshold stimulation displays a monopolar negative form and with higher voltage a preceding positivity appears as well as late components of different types

3 Differentiation of the early and late components

Several properties of the late component of the IHR in the adult sheep as well as in sheep fetuses were similar to those of the interhemispheric delayed response (IDR) in cat, described by Rutledge and Kennedy (1960) One was the marked sensitivity to asphyxia which is shown in Fig 15 illustrating the behaviour of the IHR recorded from a 110 days old fetus in which the umbilical cord was clamped As seen the late component was completely abolished at a time (1 5 min) when the initial negative wave was still present

A second difference between early and late components of the IHR was the behaviour in response to repetitive stimulation (see pp 48)

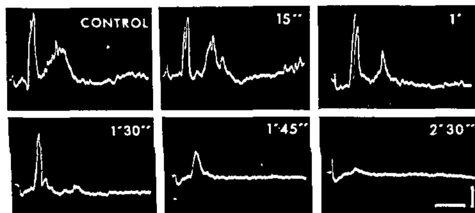


Fig 15 Effect of duration of asphyxia on the interhemispheric response in a 110 day fetus (*g frontalis superior*) Calibration 100 μV 100 msec

It has been shown that in the adult cat the IDR is mediated via extra callosal pathways in the brain stem (Rutledge and Kennedy 1961) In order to establish the callosal and extracallosal background of the early and late components of the IHR in pre and postnatal sheep the following experiments were made

Transection of the corpus callosum

Several methods for obtaining a »split brain» preparation in cats and monkeys have been described In sheep however these methods failed partly

because in this species there is only a rudimentary *falx cerebri* and partly because the *fissura interhemisphenca* is bridged over by the arachnoid. Furthermore, in the fetus the veins supplying the superior sagittal sinus are numerous and extremely fragile. The calvarium covering this sinus cannot be removed without profuse bleeding and a narrow strip of bone had therefore to be left in the midline. A favourable method of getting access to the commissures was by means of a thin and stiff steel wire which was bent at a right angle 5—8 mm from its distal end. The sharpened tip of this »instrument» was pierced through the arachnoid in the midline adjacent to the remaining bone bridge. The instrument was then gently adjusted so that its bent distal part was located along the dorsal surface of the corpus callosum which was sectioned by pressing the instrument downwards. The depth from the dorsal cortical surface to the corpus callosum had previously been measured on brain preparations from fetuses of different ages and the distances were marked along the shaft of the instrument.

The result of the surgery was checked by cutting the formalin fixed brain in the coronal plane in slices of 1—2 mm thickness which were photographed. In six fetuses, the youngest of which was 97 days, six lambs and two adults the operation was successful. At these occasions the bleeding from the midline structures was insignificant and there was little or no oedema. The anterior two thirds of the corpus callosum was found to be completely transected in all but three cases. Although in these latter cases a small part of the frontal pole of the *genu* remained intact they have been included in the material since the remaining callosal fibers probably supply only the most frontal and orbital parts of the cortex which were not systematically studied.

The stimulating and recording electrodes were placed on the cortex in the most favourable positions for obtaining an IHR with pronounced late components before the callosal surgery was performed. The spontaneous cortical activity was monitored on the oscilloscope and generally no obvious persistent change could be observed following the transection of the corpus callosum.

A typical example of the effect of sectioning the corpus callosum in a 97 days old fetus is illustrated in Fig. 16. The photograph to the right shows a coronal section of the brain in the middle part of *g. ectosylvius* after transection of corpus callosum. The responses in A, C and E were obtained before and those in B, D and F after callosal surgery. As seen in B and D the early P_1—N_1 complex (marked with arrow in C) disappeared after transection whereas the late complex was well preserved. The response in E which was obtained from *g. suprasylvius* displays an initial complex of low amplitude (at arrow) whereas the late part of the response is well developed. The extracallosal origin of the late component is fully confirmed by its persistance in F.

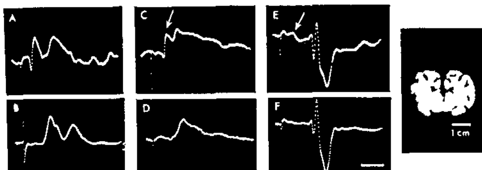


Fig 16 Effect of transection of the corpus callosum in a 97-day fetus A C and E before and B D and F after callosal section A B *g frontalis superior* C D *g ectosylvius* E F *g suprasylvius* (Initial complex of the IHR is marked with arrows in C and E) Averaged responses Horizontal bar 100 msec

A similar result of callosal transection is also illustrated in Fig 17 from three other experiments The IHR in Fig 17 A was obtained in a 15 days old lamb and both the early and late complexes were well marked The late complex was well preserved after callosal section as seen in B Records C and D in Fig 17 are from a fetus near term and those in E and F are from an adult animal *In both cases the responses were recorded from the posterior region of g frontalis superior* and both display late components consisting of a negative plateau like deflection which apparently is mediated extra callosally as judged from the postsurgery recordings in D and F

Although it is obvious that late components of the IHR persisted after sec

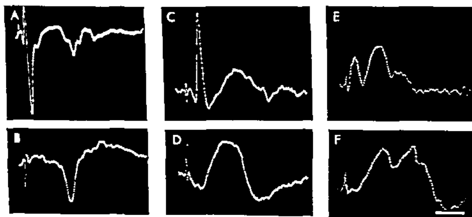


Fig 17 Effect of transection of the corpus callosum A C and E before and B D and F after callosal section A B 15 day lamb *(g ectosylvius)* C D 140-day fetus *(g frontalis superior)* E F adult sheep *(g frontalis superior)* Averaged responses Horizontal bar 100 msec

tioning of the corpus callosum their amplitude, latency, and configuration usually changed after transection (see Fig 16 A—B and 17 E—F) This change may be due to the loss of a tonic callosal input to the cortex suggested by Bremer (1966 a) Depth recordings from fetuses of 100—110 days of age showed a continuous high frequency activity indicating the presence of such an activity in the callosal fibers The finding that in many cases the late components may even be difficult to obtain after callosal transection also indicates that the activity in the callosal fibers may serve to maintain the cortical excitability This view is further supported by the observation that after callosal surgery the threshold of the extra callosal component was generally increased and the ability to follow repetitive stimulation decreased

In experiments on adult cats it has previously been demonstrated that α chloralose promotes the activation of the extra callosal interhemispheric connections (Rutledge and Kennedy 1960) In sheep, even moderate doses of α chloralose were found to induce epileptiform, high amplitude slow waves which obscured the evoked activity (*cf* Radil Weiss 1967) In order to facilitate the appearance of the late components after callosal section a combination of α chloralose (20—30 mg/kg) and a barbiturate (Thiogenal 10—15 mg/kg) was found to be helpful The result of such an experiment is illustrated in Fig 18 from a lamb in which an extra callosal late component could not be elicited after callosal section (A) unless chloralose was given (B)

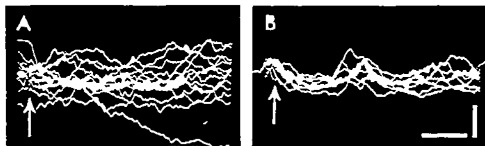


Fig 18 Enhancement of the late component of the IHR in a 70 day lamb with the corpus callosum sectioned (*g ectosylvius*) A before and B after administration of α chloralose and barbiturate Superimposed records Arrows time of stimulation Calibration 100 μV (A) 250 μV (B) 100 msec

Recording from and stimulation of the corpus callosum

In several experiments responses induced by surface cortical stimulation were recorded from the corpus callosum (CCR) by means of a glass-electrode (tip diameter about 0 3 mm) inserted in the depth of *fissura interhemispherica* Simultaneously the response from the contralateral hemisphere was recorded

The depth electrode was left in place after the experiment and during the subsequent formalin fixation of the brain The location of the tip of the electrode within the corpus callosum could be verified when the brain was sectioned in a coronal plane along the shaft of the electrode

The CCR generally consisted of a monophasic negative deflection and there were no signs of any late component The threshold of the CCR and the IHR was usually the same An example from a 98 days old fetus is shown in Fig 19 A in which the upper beam shows a CCR and the lower the IHR simultaneously recorded from the area homologous to that stimulated (*g frontalis superior*) The onset latency of CCR was generally half of that of the IHR or slightly shorter

The cortical area the stimulation of which resulted in a CCR did never comprise areas characterized by IHR s consisting solely of late components *i e* the frontal and middle portions of *g suprasylvius*

In other experiments the corpus callosum was *stimulated* directly by means of a concentric needle electrode and the surface response was recorded and compared with the IHR led off from the same cortical point The cortical response to callosal stimulation was found to have a monophasic—positive or negative—or diphasic positive negative configuration and it could be recorded simultaneously from homologous areas of both hemispheres Late components

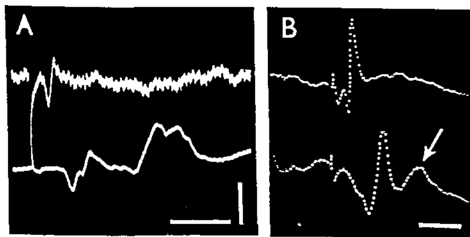


Fig 19 A Response elicited in the corpus callosum by surface cortical stimulation (CCR upper trace) and the interhemispheric response recorded simultaneously (IHR lower trace) in a 98 day fetus (*g frontalis superior*) B Surface response elicited by stimulation of the corpus callosum (upper trace) and an interhemispheric response led off from the same cortical area (lower trace) in a 128-day fetus (*g frontalis superior*) Averaged responses in B Calibration A 50 μV (upper) 250 μV (lower) and 100 msec B 50 msec

corresponding to those of an IHR, were not seen The onset latency of the response approximated half that of the IHR

The records shown in Fig 19 B are from a fetus near term in which the response to callosal stimulation (upper beam) was led off from *g frontalis superior* and displayed as a diphasic positive negative configuration The initial complex of the IHR recorded from the same position and depicted by the lower beam had a similar form but exhibited a late negative wave (arrow) which was lacking in the upper record

It was found that when a point anywhere on the anterior third of the corpus callosum was stimulated, a surface response could be obtained from almost any position of *g frontalis superior g arcuatus* and of the posterior parts of *g frontalis medialis* The scattered appearance of responses in these cases indicates that within this part of the corpus callosum commissural fibers supplying widely separated areas may be intermingled (Sunderland 1940)

In *summary* the experiments on the differentiation of the early and late components of the IHR show that the activity represented by the early P_1—N_1 complex is mediated via the callosal system whereas the late components signal activity transmitted in extra callosal pathways The two fractions of the IHR are thus equivalent to a TCR and an IDR as described in other animals In the following this nomenclature will therefore be used The results also suggest that the cortical excitability may be partly dependent on a tonic callosal inflow

4 Repetitive stimulation

The TCR was extremely labile and unable to follow repetitive stimulation when evoked in the youngest fetuses in which they could be seen (68—70 days) In order to obtain reproducible responses at this age the stimulus interval had to be very long often 20—30 seconds

At fetal ages of about 75—80 days the TCR could generally be seen to follow repetitive stimulation of a frequency of one every fifth second but at 0 5—1 Hz *there was often a definite decrease in the amplitude of the responses* An example is shown in Fig 20 A from a 78 day old fetus in which the N_1 component disappears at a stimulation rate of 1 Hz whereas the P_1 component is almost unaffected Not until a somewhat higher frequency is used (5 Hz in Fig 20 B) does the amplitude of the P_1 wave undergo a reduction The relative sensitivity of the N_1 component to repetitive stimulation in comparison with the P_1 was also apparent in older fetuses and postnatal animals

As mentioned above the IDR was found to be very sensitive to asphyxia In addition it was readily depressed by repetitive stimulation both in the fetus

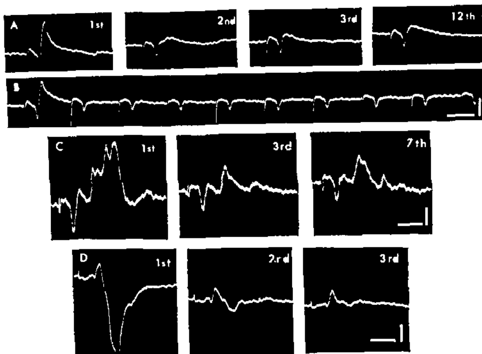


Fig 20 Interhemispheric responses with repetitive stimulation A and B 78 day fetus (*g frontalis superior*) stimulated with 1 Hz (A) and 5 Hz (B) C 100 day fetus (*g frontalis superior*) 0 2 Hz D 102 day fetus (*g ectosylvius*) 0 5 Hz Calibration 100 μV (A B and D) 50 μV (C) 100 msec

and in the mature animal In the youngest fetuses in which a significant IDR could be obtained (at the age of 90—100 days) repetitive stimulation at a rate of one every fifth second resulted in a marked reduction of the amplitude of these components (see Fig 20 C) Another type of IDR consisting of a deep positivity following upon the falling phase of the N_1 component is shown in Fig 20 D A repetition rate of 0 5 Hz resulted in a complete disappearance of the late component already at the third stimulus whereas the P_1 and N_1 of the TCR were well preserved

As mentioned above well developed IDR s were not so easily obtained in lambs and adults When present they generally did not follow repetition rates above 3 Hz without a considerable reduction in amplitude

5 Amplitude

The earliest appearing TCR—at the fetal age of 70 days—had a low amplitude which seldom exceeded 40 μV and often did not amount to more than 10—

20 μV (see Fig 21 A) At this age the spontaneous cortical activity is discontinuous and of low amplitude (Bernhard *et al* 1959) and thus did not interfere with evoked activity

At a fetal age of 90—120 days when the response generally had a constant configuration the amplitude of the IHR was regularly higher than in the more mature animal Fig 21 B shows a series of six successive superimposed records of the IHR obtained from a 102 day fetus and in this case the TCR complex was followed by a pronounced IDR (note the different amplifications in A and B) In fetuses in this phase of development the IDR component was far

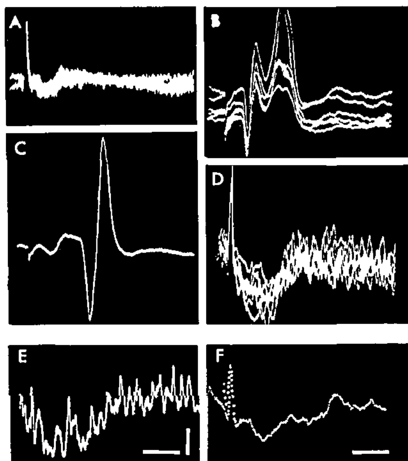


Fig 21 Development of amplitude of interhemispheric responses A 68 day fetus (fronto-parietal region) B 102-day fetus (*g frontalis superior*) C 88 day fetus (*g suprasylvius*) D adult sheep (*g ectosylvius*) E (single response) and F (averaged based on 16 responses) adult sheep (*g frontalis medialis*) Calibration 50 μV (A) 100 μV (B—E) 100 msec

more pronounced than in those near term or in lambs and adults and the ease with which a well defined IDR could be obtained from *e g g suprasylvius* in these fetuses was in striking contrast to the situation in the newborn and adult animal The response in Fig 21 C is from an 88 days old fetus and shows the presence of a huge IDR component the amplitude of which amounts to about 800 μV peak to-peak

Towards the end of the gestational period the amplitude of both the TCR and IDR became lower and it was sometimes difficult to obtain well defined responses even in the areas which were most liable to produce responses In lambs and adults the amplitude of the TCR and IDR was also often low in comparison with that of the 100 day fetus In exceptional cases it exceeded 500 μV and such a high amplitude TCR is shown in Fig 21 D obtained from the *g ectosylvius* in an adult animal In the postnatal stages the amplitude of the spontaneous cortical activity is considerable and therefore obscured the responses as shown in Fig 21 E from an adult animal in which the single response to contralateral stimulation is hardly discernable in the »noise» of the spontaneous activity The presence of a well defined TCR is revealed by the averaged response in Fig 21 F based on 16 stimulations

In *summary* in the youngest fetuses (70—75 days) the amplitude of the TCR even with supramaximal stimulation is very low (20—30 μV) With advancing fetal age the amplitude increases and during the period between 90—120 days both TCR s and IDR s appear with high amplitudes (often more than 500 μV) and consistency whereas in later stages of development as well as in the mature animal the amplitude of the responses is often considerably lower and more variable

6 Cortical distribution

When the TCR first appeared during fetal life the cortical area from which it could be obtained with focal contralateral stimulation measured about 6—8 mm With increasing fetal age this area tended to shrink In fetuses near term as well as in lambs and adults there were even difficulties in locating the re sponse Therefore the position of the electrodes generally had to be adjusted repeatedly in order to pick up a well defined response from an area which often did not exceed 1 mm²

A comparison of the responses from the focal homologous point with those recorded from the annulus shows that there was an amplitude reduction which was generally equal for all the different components of the response The onset latency of the maximal amplitude response did not significantly differ from

that of the responses led off from the surrounding area This finding suggests that the spread of activity as represented by responses obtained from the surroundings of the focal point is not due to activation of tangential intracortical pathways

In experiments on cats and monkeys it has been demonstrated that TCR s can be induced in both homologous and heterologous cortical areas (Curtis 1940 a) As judged by these results it was assumed that callosal connections to heterologous areas were more prominent in the monkey than in the cat In the sheep the occurence of TCR s in heterologous areas were exceptional and have only occasionally been observed in posterior regions of the brain in fetuses near term In general this also applies to the IDR s although this fraction of the IHR was sometimes obtained from an area which appeared to be slightly larger than that of the TCR This finding is in contrast to the observations on cats by Rutledge and Kennedy (1960) who in the adult animal observed that IDR s in response to stimulation of one and the same point may be recorded from wide spread contralateral areas

The results from the present investigation thus indicate that in the sheep the callosal connections of bilateral cortical fields are arranged in a homologous point to point manner

In the experiments to be described on the change in the regional distribution of the TCR during development care was taken to place the stimulating and recording electrodes symmetrically Supramaximal stimulation was used

In the youngest fetuses (70—75 days) the TCR was regularly found in a limited fronto-parietal area covering about ¼ of the dorsal surface The approximate extent of the area is shown as hatched in Fig 22 A (for illustrative purposes the anlage of *s suprasylvius* has been retouched in the photograph) As mentioned above the fetal brain is lissencephalic at this developmental stage and on the dorsal surface there is no indication of any future sulci It is therefore difficult to delineate the excitable area in terms of future gyri Presumably this area corresponds to the posterior part of *g frontalis superior* and the frontal part of *g suprasylvius* including *g arcuatus* Anterior and posterior to this area no responses were obtained at all nor could any TCR be obtained from the area corresponding to the future medial part of *g ectosylvius* Contrary to the TCR which was confined to a relatively small area during this phase of development direct cortical responses (DCR) could be evoked in almost all regions examined Furthermore in a 67 days old fetus in which no TCR could be found DCR s were readily induced It was also observed that the development of the areal distribution of the TCR does not seem to be related to the course of maturation of the spontaneous cortical activity since areas outside the zone of responsiveness to contralateral stimulation

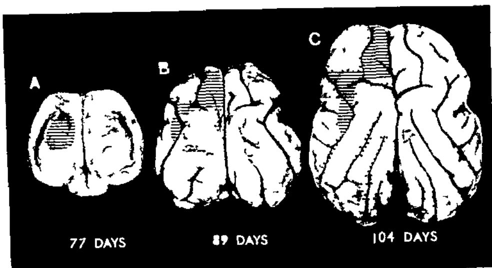


Fig 22 Distribution of interhemispheric responses in three fetuses of different ages Hatched regions from which consistent responses were readily obtained Dotted regions in which responses were inconsistently obtained (The anlage of *g suprasylvius* in the brain of the youngest fetus has been retouched)

did not necessarily display a more immature pattern of the EEG than that in which a TCR could be elicited

With advancing fetal age (75—80 days) the area capable of generating a TCR increased progressively in frontal and lateral directions and towards the age of 80 days TCR s were also be obtained from those areas which conceivably develop into the anterior part of *g frontalis superior* and the most medial part of *g ectosylvius* Furthermore responses could also be led off from more posterior portions of *g suprasylvius* As mentioned above the threshold for the TCR varied between different areas In the developing animal these variations were apparent from a fetal age of about 80 days *i e* at an age when the different cortical areas become more clarely delimited by the developing fissures Thus *e g* in a 78 day fetus the threshold for the TCR in the anterior part of *g suprasylvius* was found to be three times higher than in the adjacent *g frontalis superior* The extension of the area in which TCR s could be induced during this developmental stage is illustrated Fig 22 B The regions characterized by a relatively low TCR threshold are hatched and those in which the responses are inconsistently present and have a high threshold are dotted Not until about the 95th day could a TCR be induced in all the areas that generate responses in the adult animal The occipital part of *g suprasylvius* and the middle and frontal parts of *g frontalis medialis* were the last regions which during development displayed a TCR. The brain of a mature

fetus is depicted in Fig 22 C and the different cortical regions are marked in accordance with Fig 22 B

As described above IDR s could be found from the age of 90—100 days *i e* at a time when TCR s were present in most areas It should be pointed out that IDR s appeared at about the same time in all regions except for *g supra sylvius* where they could be induced somewhat earlier

In *summary* in the youngest fetuses TCR s are confined to an area which presumably constitutes the anlage of the anterior part of *g suprasylvius* In contrast at the same age, direct cortical responses can be induced in the larger part of the dorsal surface of the brain The area in which TCR s can be evoked extends in frontal lateral and posterior direction with increasing fetal age and at about 95 days the regional distribution of responses is similar to that of the adult sheep brain Regional difference in TCR threshold are observed from about the 80th day

IDR s appear first in *g suprasylvius* at a fetal age of about 85 days and are present in all areas from the age of 90—100 days

7 Latency of the TCR and callosal conduction velocity

The earliest appearing fetal TCR had an onset latency of its initial positive component (P_1) of 30—50 msec compared to 8—10 msec in the adult animal The corresponding values for the peak latency of this component amounted to 60—70 and 15 msec respectively Fig 23 A and 24 A illustrate the change with age of the onset (dots) and peak latencies (circles) of the P_1 component of responses obtained from *g suprasylvius* and *g ectosylvius* Fig 25 A shows the course of the onset latency curves of both the P_1 (dots) and N_1 (circles) components in *g frontalis superior*

The graphs representing the onset latency of P_1 (Fig 23—25 A dots and olid curves) show that there was a definite *increase* of about 20 msec during the first twenty days With advancing fetal age the curves slowly fall in order to reach a level equal to that of the adult animal at about one month after birth

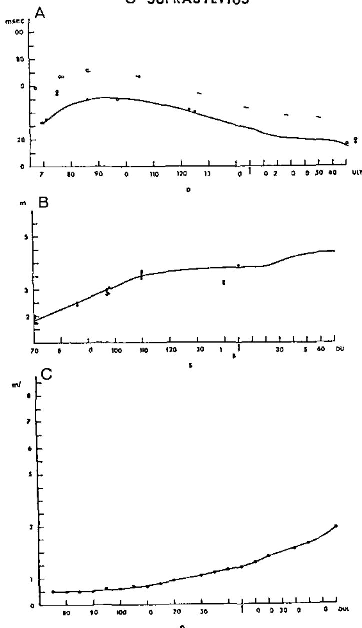
As appears from the three examples given in Fig 23—25 A the latency values varied considerably during early fetal age and with advancing matura tion there was a trend towards more constant values The latency values ob tained in adult animals were well clustered (Fig 25 A)

The change with age of the cortico cortical transcallosal distance for the three areas during development is depicted in Fig 23—25 B In view of the comparatively low validity of the measurements no corrections were made for the shrinking effect of formalin fixation For this reason and the fact that pos

sible changes in synaptic delays have not been taken into account the caculated values of conduction velocity of callosal fibers should be regarded as approximate The change in maximal conduction velocity during development for the three different areas is shown in Fig 23—25 C Fig 25 C also shows the change in conduction velocity when calculated for the onset latencies of the N_1 component (dotted curve) the values of which are given in Fig 25 A (circles dotted curve)

Between 70 and 100 days the increase of the transcallosal distance is accompanied by an increase of the onset latency This fact accounts for the initial course of the curves in Fig 23—25 C and indicates that the maximal conduction velocity of the callosal fibers during this period was almost constant at a value of about 0 5 m/sec During the next month of the gestation there was a minor increase of the conduction velocity to a value of about 1 m/sec. From the time of birth the curves for the maximal conduction velocity calculated for TCR s obtained from *g ectosylvius* and *g frontalis superior* rise more rapidly and the most conspicuous change takes place during the first postnatal month (Fig 23 C and 25 C solid curves) After this period the values increased more slowly and at about 40 days postnatal age the conduction velocity was essentially comparable to that of the adult The curve depicted in Fig 24 C represents the maximal conduction velocity of callosal fibers supplying *g suprasylvius* and in this case the curve instead displays a more continuously rising course without any sign of accelerated development during early postnatal life Obviously, there are also in the mature lamb and adult animal considerable differences in the maximal conduction velocity of fibers innerconnecting different cortical areas Thus the highest value about 8 m/sec was ultimately attained by the fibers supplying *g ectosylvius* (Fig 24 C) whereas those of *g suprasylvius* (Fig 23 C) had much lower velocity about 3 m/sec The relative magnitude of these two values probably has a good validity since the cortico cortical transcallosal distances could be measured on one and the same coronal brain slice The dotted curve in Fig 25 C stands for the development of the conduction velocity the calculation of which was based on the onset latency of the N_1 component of the TCR. When comparing the course of this curve with that of the curve based on the corresponding latency of the P_1 component of the same responses it is apparent that they differ markedly during the postnatal period This result should be considered as a possible indication of the existence of two sets of callosal fibers (see Discussion)

The latency of the IDR was found to be extremely variable In general only minor latency changes with age were found and even in young fetuses latency values of the same magnitude (75—150 msec) as those in the adult animal could be found



Figs 23—24 Change with age of A onset (dots solid curve) and peak (circles dashed and dotted curve) latency of the positive component (P1) of the TCR (*g suprasylvius* and *g ectosylvius*) B cortico cortical transcallosal distance C maximal conduction velocity of callosal fibers Curves in A are fourth order polynomial approximations to the experimental data For estimation of the maximal conduction velocity of callosal fibers the very last part of the solid curves in Fig 24 A (also Fig 25 A) has been modified to a horizontal course (dashed)

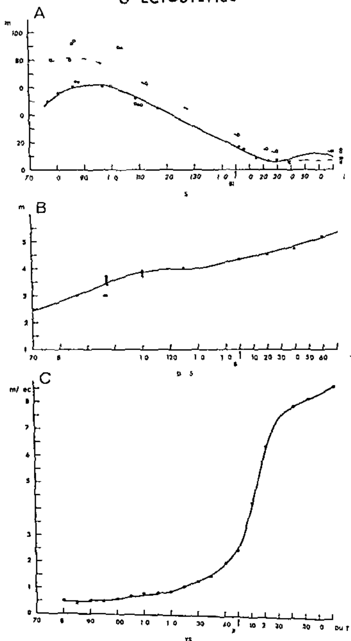


Fig 24 Legend see Fig 23

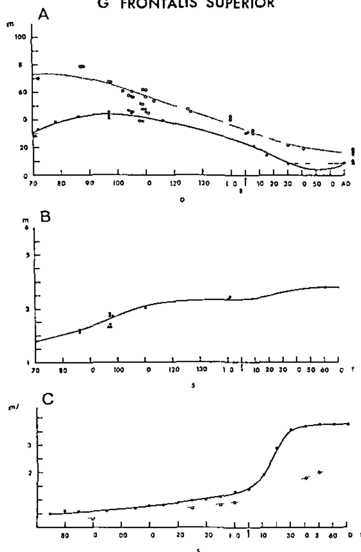


Fig ?5 Change with age of A onset latency of the positive (P1) (dots solid curve) and the negative (N1) (circles dotted curve) components of the TCR B cortico-cortical transcallosal distance C maximal conduction velocity of callosal fibers (solid curve) and conduction velocity of fibers hypothetically accounting for the negative component of the TCR (dotted curve) Construction of curves in A see legend to Fig 23

In *summary* the onset latency of the TCR in the youngest fetuses is 3—5 times that in the adult animal During the first month there is a slight *increase* of the latencies followed by a slow decrease until adult values are attained at about 30 days postnatal age The maximal conduction velocity of the callosal fibers undergoes a substantial increase primarily during the first month after birth During later stages of maturation considerable regional differences are seen in maximal conduction velocity of callosal fibers interconnecting different cortical areas

MYELINATION OF THE CORPUS CALLOSUM

The first signs of an incipient myelination of the forebrain was observed as an increasing gliosis taking place in fetuses between 95 and 105 days of age In addition to an increase in the number of glial cells their nuclei also became more heavily stained The gliosis preceding the appearance of stainable myelin (*cf* Peters 1960 Chevreau and Marty 1961) was first seen along the deeper portions of the fiber tracts which connect the cortex with subcortical structures such as the thalamus A specimen taken from this region in a 97 days old fetus is shown in Fig 26 A to illustrate the abundance of glial cells with heavily stained nuclei and arranged in rows (*cf* Fleischhauer 1967) As is illustrated in Fig 26 B lightly stained myelin could be demonstrated in the deeper parts of this fiber system (marked R) at a fetal age of about 110 days At this stage of development the corpus callosum (CC arrow) remained unstained Not until the end of the fetal period *i e* 130—140 days the corpus callosum was slightly stained indicating the beginning of myelination There was, however a considerable difference between the degree of staining of this structure and of the radiating stalk fibers

The myelination of the corpus callosum is also illustrated in detail in Fig 27 A—H All sections depicted were taken from a paramedial portion of corpus callosum adjacent to *g cinguli* The histographs depicted in Fig 27 A and B are from two fetuses of about 120 days of age In A there is no stainable myelin to be seen and there is gliosis primarily near the dorsal border of the corpus callosum In B however the very first traces of myelin can be seen The glial cells are concentrated to the dorsal and ventral parts of the medial portion and there is also a thin bundle of myelinated fibers which is orientated obliquely and runs in a lateral dorsal to medial ventral direction (arrow) In close connection to this bundle a marked gliosis can be seen The orientation of this oblique tract indicates that it represents the cingulo striate decussating system which has been described in rodents (Cajal 1955) monkey

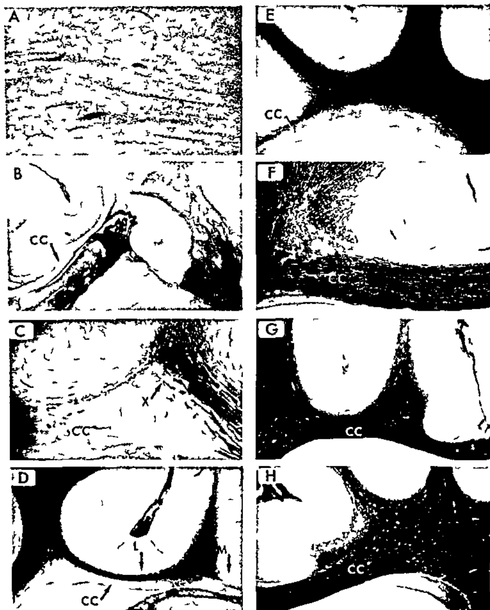


Fig 26 Myelination of callosal *(C C)* radiating *(R)* and stalk fibers Coronal sections of the brain A gliosis as a sign of incipient myelination of radiating fibers in a 97 day fetus Fetuses of 110 (B) 137 (C) and lambs of 2 (D) 15 (E) 24 (F) 70 (G) days of age and adult sheep (H) In C letter X denotes intersection between callosal and stalk fibers In D letters *L* and *M* denote lateral and paramedial portions respectively of the callosal system (Loyez stain)

(Locke and Kruper 1965) and man (Locke *et al* 1964) In recent studies on rat (Jacobson 1963) and man (Yakovlev and Lecours 1967) it was found that these »corpus callosum perforating» fibers possess myelin earlier than the rest of the callosal fibers

The difference in staining between the cingulo striate bundles and the neocortical commissural fibers located in the core of corpus callosum (Locke *et al* 1964) was more apparent later in development as seen in Fig 27 C from a 137 days old fetus At this age faintly stained myelin also appeared in the rest of the corpus callosum (*cf* Fig 26 C)

At full term (145 days) the core of the corpus callosum has become faintly stained which reflects the beginning of myelination of the neocortical commissural systems (see Fig 27 D) At the same time the fibers of the white matter in the cortical lobuli were heavily stained As illustrated in Fig 26 C and D lightly stained callosal fibers penetrate into the dark lobular bundles (\, arrow) Myelination of the bulk of callosal fibers primarily took place during the first two or three weeks after birth As seen in Fig 27 F the callosal fibers are deeply and homogeneously stained in a 24 day lamb and at 70 days of age (27 G) the process of myelination appears to be completed When comparing the degree of staining of the fibers in the cortical stalk with that of corpus callosum (Fig 26 G and H) it also appears that the myelination of the corpus callosum in a 70 days old lamb is of the same order as that of the adult sheep

In the intermediate stage of myelination during the first postnatal week, when local differences in the intensity of staining may be most easily discerned the degree of staining was found to be essentially the same in the medial and lateral portions of the callosal fibers (see Fig 26 D arrows marked M and L resp)

Comments

The results obtained in the present investigation are in general accordance with those presented by Romanes (1947) in a study on the prenatal myelination of the central nervous system in sheep Thus, *e g* the first appearance of faintly stainable myelin in the thalamo-cortical radiating fibers at the age of about 110 days (*cf* Fig 26 B) has been confirmed in the present study According to Romanes the beginning of myelination of the corpus callosum occurs later than in all other forebrain structures examined However, Romanes did not pay attention to the early myelination of the cingulo-striate transcallosal system which begins 20 days before that of the core of the corpus callosum.



Fig 27 Myelination of callosal fibers (paramedial portion) Coronal brain sections from fetuses of 117 (A B) 137 (C) and lambs of 1 (D) 15 (E) 24 (F) and 70 (G) days of age and from an adult sheep (H) Arrow in B cingulo-striate fibers (Loyez stain)

The large neocortical commissural system is far from mature in the newborn sheep. The delayed development of this system is especially striking when compared with that of the neocortical stalk fibers in which the myelination is almost completed at birth. On the other hand it should be taken into account that the »cycle» of myelination in corpus callosum in sheep is only one to two months which is short in comparison with that in *e.g.* man which is not completed before puberty.

The finding of a late appearance of myelin in corpus callosum seems to be common for all species which have been investigated. Thus, *e.g.* in both cat and rat myelination does not start until 14 days (references, see Jacobson 1963) and in rabbit at 4 days after birth (Ulett *et al.* 1944). According to most authors myelin appears in corpus callosum during the second postnatal month in man (*e.g.* Yakovlev and Lecours 1967) whereas Luttenberg (1966) claims there are some myelinated fibers to be found already at the 30th prenatal week.

Corpus callosum has been regarded to rank high in the evolutionary order of the different parts of the brain (*e.g.* v. Mihalkovics 1877, Abbie 1939) and the late appearance of myelin in corpus callosum as compared with other structures in the telencephalon is consistent with the view that the acquisition of myelin in different structures of the brain proceeds in the order of their phylogenetic age (*cf.* Flechsig 1927). The early appearance of myelin as well as the short cycle of myelination in the limbic cingulo-striate transcallosal system in comparison with the supralimbic commissural system also favours this view. According to some authors myelin appears earlier in nerve fibers which in the mature stage acquire large diameters than in those which remain thin (discussion see Hess 1954). The fact that the majority of fibers in corpus callosum are thin $< 3\mu$ (Tomasch 1954, Luttenberg 1965, Fleischhauer and Wartenberg 1967) would thus be consistent with its late myelination.

In a study on the myelination of the corpus callosum in man de Villaverde (1919) found that the middle portion which is located in the coronal plane of the »Zentral windlung» aquires myelin earlier than the *genu* and *splenium* whereas Luttenberg (1966) observed myelinated fibers to appear first in the *genu*. Yakovlev and Lecours (1967) on the other hand, claimed that myelination of the corpus callosum proceeds in a posterior-anterior direction. A comparison of the staining in different coronal sections made in the present study suggests that the anterior portions of the corpus callosum become myelinated somewhat earlier than the posterior parts.

Mingazzini (1922) on the basis of a study in man states that the medial portions of the callosal fibers are later myelinated than the lateral, an observation which speaks in favour of either a cellulofugal or a cellulopetal acquisi-

tion of myelin in the fibers (discussion, see Marty 1962) In the sheep no significant difference in the staining between the medial and lateral parts was found in sections of the brain in plane with the course of callosal fibers This observation indicates that the process of myelination of the callosal fibers in sheep takes place *in toto*

In *summary*, in the sheep myelin within the corpus callosum first appears in the cingulo striate decussating system at about 120 days of fetal age Myelination of the commissural callosal system located in the core of the corpus callosum is late in comparison with other forebrain structures and takes place primarily during postnatal life The apposition of myelin probably occurs simultaneously in the proximal and distal portions of the callosal neurons The process of myelination of the corpus callosum seems to be almost completed at about 40 days after birth

DISCUSSION

Interhemispheric responses

The results from experiments on the differentiation of the two fractions of the IHR show that the initial P_1—N_1 complex is mediated via the callosal system and thus corresponds to the transcallosal response (TCR) described in other animals They furthermore reveal that the late components have an extra callosal background and are equivalent to the interhemispheric delayed response (IDR)

In the *adult* sheep TCR s predominate in the motor and primary sensory areas whereas in other species—particularly in the cat—such responses have been reported to be most easily obtained in association areas (*e g* Curtis 1940 a) Of special interest is the prominence of responses in the sheep s *motor* cortex characterized by the presence of giant pyramidal cells and confined to *g frontalis superior* In the ungulates a comparatively large part of the motor area represents the muscles of the lips and the neck (Breazile *et al* 1966) whereas in *e g* the cat the limbs have more extensive representation In view of the observation that within the motor area of the monkey and the cat TCR s are primarily obtained from regions of trunk and face projection (Curtis 1940 a McCulloch and Garol 1941) the ample supply of commissural connections to *g frontalis superior* in the sheep is not unexpected According to cytoarchitectonical studies of the sheep s cortex large pyramidal cells are less abundant in the posterior part of *g frontalis superior i e* behind *s splenialis* than in the

middle part (King 1911 b Simpson and King 1911) This posterior region which is known to represent the hindlimb muscles generally exhibits TCR s irregularly and with a variable configuration There also seems to be a correlation between the presence of large pyramidal cells and a high excitability to contralateral stimulation in other areas Thus, pronounced responses were obtained in *g arcuatus* which according to Bagley (1922) is characterized by the presence of giant pyramidal cells and therefore was included in the motor area of the sheep It has also been observed that destruction of this region—called area 6 by Bagley—causes degeneration in corpus callosum and in the contralateral cortex Marked differences in the occurrence of the TCR were found between *g suprasylvius* and *g ectosylvius* Although these regions have many cytoarchitectonic features in common large pyramidal cells are less abundant in *g suprasylvius* than in the adjacent *g ectosylvius (regio occipitalis* and *parietalis* respectively Rose 1942)

Adrian (1943) found that an exceptionally large proportion of the *somesthetic* area (I) in the sheep receives projections from the lips (see also Woolsey and Fairman 1946) The lip area which in the sheep is located in the posterior lateral part of *g frontalis medialis* is the only area of this gyrus in which a TCR could be easily obtained This finding is consistent with the above mentioned observation (p 17) by Ebner and Myers (1965) on the distribution of callosal fibers in different parts of the somesthetic area in the cat and the raccoon

Rutledge and Kennedy (1960) described the IDR in the adult cat as a predominantly positive deflection following upon the negative component of the TCR with variable delay IDR s were found to be present primarily in *g suprasylvius* In the current study this type of IDR response has been observed to occur mainly in *g suprasylvius* and *g arcuatus* whereas a late component in the form of a negative long lasting potential was most prominent in the sensory motor areas Since this negative response remained after callosal section it has been considered to be a variant of the IDR A late IHR component appearing as a negative plateau has been observed in adult rabbits by Eidelberg (1963) who also claimed that it is mediated via extra callosal pathways. It was not mentioned whether the occurrence of this type of response in the rabbit was confined to any particular area In the adult sheep the IDR appeared irregularly and was often absent unless facilitated by the use of drugs However experiments on prenatal animals provided consistent IDR s and it was found that these responses were particularly well pronounced within the parietal association area *i e* the frontal and middle part of *g suprasylvius* In contrast to the activity mediated via corpus callosum the distribution of the response mediated by extra callosal pathways is thus similar to that found in

the cat by Rutledge and Kennedy (1960) It should however, be emphasized that in the sheep IDR s were also found in the primary sensory motor areas

In the *fetal* sheep the first appearance of a TCR coincided with the appearance of the direct cortical response (DCR) which is also first obtained at an age of 68—70 days (Eidelberg *et al* 1965) In the cat both the DCR (Purpura *et al* 1960 b) and the TCR (Grafstein 1963) have been shown to be present at birth Since these and other previous studies (*e g* Poon 1965) were confined to the postnatal period the very first developmental phase of the responses was not studied The first appearance of a DCR denotes the developmental stage at which the cortex becomes excitable to surface electrical stimulation This event in the cortical functional development presumably accounts for the fact that TCR s may first be elicited at this same age At the stage of the first appearance of TCR and DCR the electrical excitability of the cortical neurons differs from that of the more mature animal by the exceedingly high threshold In a study performed on the developing postnatal rat the TCR threshold was shown to be very high in the neonatal period (Hatotani and Timiras 1967) and furthermore Hines and Boynton (1940) found that a much higher current was required to induce movements by cortical stimulation in the newborn than in the adult monkey (see also Noback *et al* 1962) On the other hand, Grafstein (1963) did not find any difference in TCR threshold between the neonatal and mature cat there are no data available on the prenatal stages of this animal

There are several characteristics of the immature central nervous system which may account for its relatively low excitability to electrical stimulation One possible explanation would be that the membrane of the immature neuron has properties fundamentally different from those of mature neurons as has been recently suggested by Purpura *et al* (1965) There is also the possibility that the small surface area of the immature neuron with its primitive dentritic tree is a limiting factor for electrical excitation (*cf* Schade *et al* 1964) During the first week after the appearance of the TCR its threshold steadily decreases and during the same time there is a remarkable growth of the dendrites The high threshold of the TCR may also be ascribed to functional immaturity of the synaptic mechanisms involved Finally one cannot exclude the possibility that the comparatively extensive extracellular space as well as the high content of water in the immature cortex constitutes a shunt for the applied electrical current which would be less effective as a stimulus for the excitable tissues (*cf* Sedlacek and Macek 1966)

The fact that within the primary sensory area both evoked responses to tactile stimulation (Molliver 1967) and DCR s (Eidelberg *et al* 1965) can be obtained at an earlier age than TCR s indicates that the cortex may be both

directly and synaptically excitable a long time before cortical stimulation is followed by transcallosal responses Since the same cortical neurons may be activated both by thalamo cortical and transcallosal fibers (Latimer and Kennedy 1961) the late appearance of the TCR in the somatosensory area seems to be due to a delayed maturation of the commissural system of this region

There are no functional data given in the literature on the development of the distribution of the callosal connections In his morphological study Auroux (1964 a, b) found that the frontal areas were the first to establish bilateral connections via corpus callosum and in the present investigation TCR s were first observed to appear in a limited fronto-parietal cortical area At this stage the fetal brain is lissencephalic and it is difficult to define this cortical area in terms of future convolutions There is also the possibility of an anatomical redistribution of the different cortical areas with increasing convolution as demonstrated by Hines and Boynton (1940) One may assume however that the area in which a TCR was first obtained eventually develops into the anterior and middle parts of *g suprasylvius* together with the posterior portion of *g frontalis superior*

The progressive extension in lateral direction of the area exhibiting transcallosal responses is in accordance with the morphological data presented by Auroux (1964 a b) Thus provided that most callosal fibers interconnect homologous cortical areas as indicated by the results of the present study a centripetal formation of the corpus callosum implies that fibers ultimately meant for the lateral areas require a longer period in order to reach the contralateral cortex than those which establish interconnections between more medial areas Accordingly *gg suprasylvii* exhibit TCR s earlier than *e g gg ectosylvii* although the irregular appearance of responses within *g suprasylvius* in the mature animal indicates that this area ultimately will have a poor commissural supply

The relatively late appearance of the TCR in all posterior regions of the brain is somewhat unexpected when considering Luttenberg s assumption (1965) that corpus callosum is formed by intussusception If that were the case TCR s should at least occasionally be found in posterior areas of the cortex also during the earliest phase of development On the other hand the absence of responses within these regions at an initial stage of TCR development may be due to a delayed functional maturation of the posterior cortical regions as suggested by the pattern of EEG displayed (Bernhard and Meyerson 1968 *cf* Verley 1959) also the cortical cytoarchitecture of these regions displays a later maturation than that of more anterior areas (Marty 1962)

In the youngest fetuses the TCR consists solely of or is dominated by a surface positive deflection This has recently been confirmed in a study on neonatal rabbits (Verley personal communication) In Grafstein s (1963) investi

gation the transcallosal response in kitten exhibited the typical positive negative configuration of the adult animal already at birth

It has been claimed that the presence of an evoked surface-positive potential reflects a comparatively advanced stage of functional maturation of the neo cortex (*e g* Ellingson 1964) This view is based upon a number of studies in which evoked potentials in response to tactile and visual stimulation have been found not to display positive deflections until later stages of development (*e g* Scherrer and Oeconomos 1954, Purpura 1961 a Marty 1962) However, an acoustic stimulation is followed by a response with a positive negative confi guration at its first appearance in the developing animal (Rose *et al* 1957) and cortical responses elicited by midthalamic stimulation may also display surface positivities even in the newborn kitten (Purpura 1961 b) All these stu dies were confined to the postnatal period of development

The somesthetic evoked response which is already present at birth in a given species (cat rabbit) has only recently been studied in the earliest phase of its development In the *fetal* sheep it has been found that the response induced by tactile stimulation of the nose is present already at 50 days of fetal age and that at this early stage it is purely positive (Mollıver 1967) Later in develop ment a subsequent negative deflection develops and the positivity successively decreases At about 80—85 days of fetal age the somesthetic response displays a configuration—*i e* monophasic negative—similar to that found in newborn kittens and rabbits By comparison the maturation of the neocortical structures of a newborn kitten (as judged from Golgi preparations) corresponds more closely to that of a sheep fetus of 70 instead of 80 days, a difference which is critical during this particular phase of development (Åstrom 1967) The dis crepancy between the findings from the cat and the sheep thus warrants cau tion in deducing functional properties from morphological data unless supple mented by correlative evidence

The characteristics of both the TCR and the somesthetic evoked response in the fetal sheep support the view that surface positivities may be obtained even in an immature neocortex

There are several reasons for also considering the development of the som esthetic evoked response in a tentative analysis of the neuronal events respon sible for the change in pattern of the TCR during maturation Firstly, the sequence of developmental changes of the form of the two responses is very similar during the initial phases of their early ontogeny Secondly in the adult animal the general configuration of the two responses is essentially the same The mode of their generation within the cortex has also been assumed to be similar (Amassian 1961) and it has been shown that transcallosal and som

esthetic afferent terminals may impinge on the same neuron or pool of neurons (Latimer and Kennedy 1961, Dubner and Rutledge 1965)

The fact that both the TCR and the evoked response to tactile stimulation are positive at their earliest appearance suggests they are generated by similar neuronal mechanisms in the cortex A surface positive deflection may be a sign of a depolarization in the depths of the cortex or of a hyperpolarization in a locus close to the recording electrode, i e in the superficial layers of the cortex For reasons to be discussed below it is not likely that surface positivities in an early stage of maturation signal postsynaptic hyperpolarization and therefore excitatory processes in the deep layers probably account for the positivities of both types of responses during the initial phase of the development Inhibitory processes as generators for the positive deflection of the immature TCR are furthermore made less probable by the fact that this response in the adult primarily represents deep seated EPSP s (Latimer and Kennedy 1961 Ajmone Marsan and Morillo 1963)

Preliminary results from a forthcoming study of the development of the somesthetic response indicate that in the youngest fetuses a subcortical depolarization of the afferent terminals may be the structural and functional correlate for the surface positive deflection of that response (Meyerson and Persson in preparation) Similarly the immature positive TCR may be due to depolarization of the ingrowing callosal terminals On the other hand it may signal deep postsynaptic excitatory processes According to Åstrom (1967) the cells in the middle pyramidal stratum of a 70 days old fetus are relatively well developed and their »apical extensions are thicker (than those of a 65 day fetus) and they have branches proximally and even indications of spines» This observation should be considered in view of a recent finding that the synaptic junctions between callosal terminals and pyramidal cells are predominantly located on the oblique branches of the apical dendrites (Globus and Scheibel 1967) It is also noteworthy that at the age of about 70 days stellate cells appear for the first time within the deep pyramidal layer and their ramifying extensions could make them serve as interneurons facilitating intracortical connections The functional role of stellate cells which in the mature neocortex amount to 25 % of the total population of neuronal cells, has recently been emphasized (Scheibel 1962 Schade *et al* 1964)

Finally the possibility that antidromic activation of the callosal fibers may contribute to the surface positivity could also be considered However the occurrence of antidromic activation is unlikely in the immature fetus since it is regarded to be of no significance for the TCR in the adult (Curtis 1940 a Latimer and Kennedy 1961)

At the age of about 80 days both the TCR and the somesthetic response

begin to display a more constantly appearing negative deflection subsequent to the initial positivity This coincidence is certainly a challenge to assume a common causal factor although no dramatic changes may be seen in the morphological features of the cortex at this age Grafstein (1959, 1963) has advanced the view that the positive and negative phases of the TCR are independently generated by two sets of callosal fibers which differ from each other in *e g* the rate of myelination (*cf* von Euler and Ricci 1958) A similar idea is found in a discussion by Purpura *et al* (1964) on the changing configuration of the somesthetic response in the postnatal cat The finding in the present study of a differential development of the positive and negative phases of the TCR as well as the differential course of the development of the conduction velocities when calculated separately for the two components, is compatible with Grafstein s view The most likely cause, however of the occurrance of a surface negative deflection of both the TCR and the somesthetic response would be the progressive functional maturation of excitatory postsynaptic processes in superficial cortical strata taking place during the period between 80 and 90 days The fact that the negative component of the TCR was easily blocked even by low frequency repetitive stimulation indicates that it has a postsynaptic background Whether this synaptic activation takes place at the site of stimulation or recording or both cannot be deduced from the present results

During the 20—25 days after its first appearance the amplitude of the TCR in the fetal sheep steadily increased The same has also been found to occur in kittens during the first 20 days after birth and was ascribed to the growing »receptor area» at the cortical neurons as well as to a supposedly more efficient synaptic transmission (Grafstein 1963) Similar findings have also been made in studies on evoked response to afferent stimulation (*e g* Scherrer and Oeconomos 1954 Marty 1962) In the fetal sheep, evoked somesthetic responses first appear at about 50 days of age and are of low amplitude but in fetuses of 80 days they may attain peak to peak amplitudes of 2 mV (Meyerson and Persson in preparation) In more mature preparations however responses of such high amplitudes are never obtained under comparable conditions and towards the end of gestation when both the somesthetic response and the TCR were found to appear more irregularly they generally had a much lower amplitude than 30—40 days earlier The fact that Grafstein never observed such a reduction of the TCR amplitude during later stages of maturation is probably due to that she used *cerveau isolé* preparations since it has been experimentally demonstrated that the amplitude and consistency of IHR s may be greatly modified by influences originating from the brain stem Thus in the adult cat high frequency stimulation of the brain stem reticular formation causes a stabi

lization of an otherwise variable TCR (Purpura *et al* 1960 a) It has further been shown that the IDR is highly dependent upon the state of sleep or wakefulness being most prominent during synchronized sleep (Baldissera *et al* 1966) and an arousal reaction is followed by a reduction of the TCR amplitude by 60—90 % (Favale *et al* 1964) The observation in the present study that in the fetus the IDR was augmented when induced concomitantly with periods of spindling activity is consistent with these last mentioned findings (*cf* Moruzzi *et al* 1950) Similarly an earlier observation that arousal reactions can not be induced in the fetal sheep until near term (Bernhard *et al* 1959) is of interest in consideration of the inconsistent appearance of IDR s from the age of about 120 days

The regular appearance of a high amplitude IHR in fetuses around 100 days of age may depend on the lack of inhibitory influences Such a notion is suggested by the finding that chloralose promotes the appearance of IDR s in adult animals (Rutledge and Kennedy 1960) since this drug is generally considered to have disinhibitory effects (references see Bava *et al* 1966) The same conclusion can be deduced from the observation that TCR s are enhanced by topical application of strychnine (Curtis 1940 a Chang 1953 a), since this substance has recently been shown to depress inhibitory processes also in the neocortex (references see Eccles 1966) Thus the decrease of the consistency and amplitude of both fractions of the interhemispheric response during the late part of gestation may reflect a progressive maturation of inhibitory mechanisms

A later maturation of inhibitory than of excitatory synaptic mechanisms in the neocortex is supported both by morphological and electrophysiological investigations According to Voeller *et al* (1963) axo-dendritic synaptic structures could be demonstrated in electron microscopic preparations in the newborn cat whereas synapses on the soma—presumably inhibitory— were not seen until later Correlative electrophysiological evidence has been provided by Purpura (1961 c) who found a delayed functional maturation of inhibitory relative to excitatory synaptic processes (*cf* Anggard *et al* 1961 for the spinal cord) The relatively late development of neocortical inhibitory synapses has also been assumed by Huttenlocher (1967) The finding that in the developing sheep the TCR aquires an essentially adult pattern already at about 80 days of fetal age is consistent with the fact that excitatory synaptic structures presumably are present from a comparatively early stage of cortical maturation and that the TCR in the adult animal predominantly represents excitatory axo-dendritic activation (Peacock 1957 Landau *et al* 1961)

In the developing fetal sheep the reduction of the amplitude of the TCR is accompanied by a decrease of the cortical surface activated as indicated

by the great difficulty of locating a response in the older fetuses and post natal animals Actually Grafstein (1964 b) found the space constant of the TCR to diminish with advancing age of kittens and the same has been de monstrated for the development of the visual response (Marty *et al* 1958) This developmental process is evidently not dependent upon the maturation of influences from the brain stem, since in Grafstein s *cerveau isole* preparations they were eliminated The decreasing area of the cortical surface involved in the generation of the responses as well as the reduction of their amplitude may therefore be a result of a change of the spatial organization of excitatory and inhibitory mechanisms within the cortex The maturation of inhibitory elements surrounding a core of neurons excited by transcallosal stimulation (Asanuma and Okuda 1962) may constitute a background for the formation of functional cortical columns and might account for the progressive spatial restriction of the response with development

The exceedingly rapid decline of responses during repetitive stimulation of immature nervous structures has been repeatedly documented (*e g* Scherrer and Oeconomos 1954 Molliver 1967) Although it can also be observed in the peripheral nerve (Garcia Austt *et al* 1956) this characteristic of early stages of functional nervous development is most apparent in systems comprising polysynaptic transmission as illustrated by the differential effect of repetitive stimulation on TCR and IDR Grafstein (1964 b) has discussed this phenome non in terms of data on the functional properties of the immature neuromus cular junction and assumes that long lasting PSP s increased and generalized sensitivity to transmittors and susceptibility to presynaptic blocking may all be factors of importance for the inability to respond to repetitive excitation Later on Purpura *et al* (1965) has shown that PSP s of immature neocortical neurons actually have exceptionally long durations and he concludes that the »fatiga bility» during repetitive stimulation is partly accounted for by the presence of such long lasting inhibitory events

The functional development of extra callosal interhemispheric connections as represented by the IDR has not been studied earlier In the current investi gation it was shown that IDR s could not be observed until considerably later in development than TCR s due probably to the complexity of the extra callo sal pathways (Rutledge and Kennedy 1961) Although involving totally differ ent pathways the TCR and IDR seem to be functionally interdependent as indicated by the fact that they may both activate the same cortical neuron (Latimer and Kennedy 1961 Dubner and Rutledge 1965) A finding along the same line in the present study was that callosal section is followed by a considerable increase of the IDR threshold as well as by changes of its con figuration which may be explained by the loss of callosal tonic activity (»dy

namogenese commissurale tonique» Bremer 1966 b, see also Berlucchi 1965)

A salient feature of the IDR both in young fetuses and mature animals is its low safety factor as manifested in *e g* the extreme sensitivity to repetitive stimulation In particular it should be noted that the IDR in early stages of its development is highly susceptible to asphyxia in contrast to the direct cortical response and—to some degree—also the TCR which has been shown to be remarkably resistant (Meyerson 1964 see also Eidelberg *et al* 1967)

One of the most conspicuous features of evoked potentials during early stages of ontogeny is their remarkably long latencies This phenomenon has been repeatedly demonstrated in different species and is a common characteristic of both central and peripheral nervous structures The rate of change of latency with development may however differ as to the type of evoked activity and the length of the maturational cycle in different species is also of importance as has been reviewed by Marty and Scherrer (1964)

The initial phase of increasing latencies of the TCR concomitant with an increasing transcallosal distance implies that the increased conduction *distance* is not compensated for by a simultaneous increase of the conduction *velocity* and illustrates the changing relations during development between conduction time velocity and distance The functional significance of this for the maturation of central nervous integration has been recently discussed by Scherrer *et al* (1967) Similar results have not been reported by others and it is conceivable that the prolonged maturation of the sheep is a prerequisite for disclosing such a developmental sequence which might be concealed in animals with a more limited period of premyelin ontogenesis The fact that the changes of cortical thickness relative to the overall transcallosal distance have not been accounted for can hardly be of any significance in explaining the increasing values of latency neither could the shrinking caused by formalin fixation whose effect is not likely to change during this short developmental period

It is common knowledge since the classical studies by Tilney and Casamajor (1924) that conducted activity can occur in fibers prior to their myelination a finding that was confirmed also for callosal fibers (Ulett *et al* 1944) These fibers show an exceptionally prolonged period of excitability before the beginning of myelination because of their delayed morphological maturation

The maximal conduction velocity of the callosal fibers in the youngest fetuses was estimated to about 0 5 m/sec, a value comparable to that found by Grafstein (1963) in neonatal kitten (*cf* Verley 1967 a) Similarly to the results obtained on kitten (Grafstein 1963 1964 a) there was a slight increase of the conduction velocity during a period before any stainable myelin could be traced in the corpus callosum and at about 110 days of age the velocity

ounted to about 1 m/sec Such an increase in the conduction velocity can, course, be due to the very beginning of myelination of the callosal fibers in ir cellulo-proximal or cellulo-distal segments, where there is no possibility to erentiate these fibers from those which are already myelinated It is, how r, unlikely that the myelination process of the callosal system should pro- d in two phases an initial one with a cellulo-fugal or cellulo-petal aquisition myelin and a later one during which the myelination takes place *in toto* e increased conduction velocity prior to myelination could instead probably ascribed to an increase of the fiber diameters Electronmicroscopic study corpus callosum in the kitten has recently demonstrated that the callosal ers which have an average diameter of 0 25 μ in the immediate neonatal iod do not acquire myelin until the diameter has increased to about 0 6 μ leischhauer and Wartenberg 1967)

Concomitant with the first appearance of stainable myelin within the core the corpus callosum there was a relatively marked increase of the conduction ocity and adult values were attained at about 1½ month postnatal age It iardly possible to state definitely the age at which the myelination process is npleted since it may continue in the thinnest fibers without contributing to over all intensity of the staining in sections prepared for the light micros py (*cf* Fleischhauer and Wartenberg 1967) Since the estimation of the nduction velocity was based upon the shortest latency obtained *i e* the onset ne for the initial positivity (P_1) changes in the thinnest fibers are of no nificance in this context Considering the slow time course of the transcallo activation the fraction of the delay due to synaptic transmission within the rtex could hardly significantly influence the general feature of the develop ent described

A dramatic decrease of the TCR latency occurring almost over night between 27th and 28th postnatal day in kitten was reported by Grafstein (1963) is finding could not be confirmed in the present experiments and may be ex ined by the protracted functional and morphological development in the eep On the other hand the abrupt change of the latency in the cat was tually shown to be caused by the sudden appearance of an early positive flection which was ascribed to the accelerated myelination of a set of callosal ers with comparatively large diameters The definite absence of such an vious change of the configuration of the TCR in sheep may tentatively be ferred to as a species difference in cortical organization of callosal fibers

The regional differences in the abundance of callosal fibers that has been scussed above seem to be related to the magnitude of the conduction velo- ties Thus areas in which there is an ample supply of callosal fibers (*e g* ie medial parts of *g ectosylvius*) as judged by the characteristics of the TCR

are apparently interconnected by means of relatively large fibers as revealed by the higher conduction velocities found for these areas Evidence based on the change of distribution of TCR s during development indicates that callosal fibers which ultimately acquire a relatively large diameter do not grow faster which implies that they do not reach their destination earlier than those which remain thin

B BILATERAL SYNCHRONY OF SPONTANEOUS CORTICAL ACTIVITY IN ONTOGENY

1 Electrocortical activity of the intact preparation

The term bilateral synchrony of the EEG is generally used to denote the relatively simultaneous occurrence of events in the two hemispheres with reference to the peaks of the individual waves (*cf* DeLucchi *et al* 1962) Since the early fetal EEG which is the main concern of this part of the investigation is discontinuous bilateral synchrony as a sign of interhemispheric coordination will refer both to the phase relationship of the EEG waves and to the simultaneous bilateral appearance of epochs of activity The former type of synchrony is preferentially studied by cross correlation analysis

In the original study of EEG development in the fetal sheep spontaneous cortical activity was first observed at a gestational age of 65 days (Bernhard *et al* 1959) Extended investigations with improved methods of recording have later proved activity to be present somewhat earlier at 60 days of age corresponding to fetal weights of about 50 grams (Bernhard *et al* 1967 Bernhard and Meyerson 1968)

In fetuses between *60 and 65* days of age the electrocortical pattern is less well defined than during the following stage of development The activity was discontinuous and consisted primarily of slow waves with a polyphasic configuration having a total duration of about 0 5 second The electrically »silent» intervals between these waves varied between 5 and 20 seconds The slow waves were often clustered forming short periods of activity of polymorphic appearance (see Fig 28 A)

The fact that the slow wave activity disappeared about 1 min after asphyxiation of the fetus shows that it has a physiological background (*cf* Eidelberg *et al* 1967) During this earliest phase of EEG development rhythmic activity was rare but may sometimes be seen as short bursts of 6—8 Hz diphasic waves which generally are superimposed upon the slow wave activity When recording with a shorter time constant the spindle shape of this periodic type of activity becomes more prominent as illustrated in Fig 28 B

In the youngest fetuses there were no regional differences in the pattern although the activity seems to be most prominent in the parietal areas of the still lissencephalic brain

Because of the sporadic occurrence of the slow wave activity in fetuses during this phase it was not feasible to perform automatic analysis of its bilateral synchrony Visual examination revealed that there was a remarkably high incidence of simultaneous bilateral activity when led off from symmetrical areas An example from a 60-day fetus is depicted in Fig 28 which illustrates the presence of a certain degree of bilateral synchrony both of slow waves (Fig 28 A left) and of an epoch of polymorphic activity (Fig 28 A, right) The activity was led off with a bipolar configuration between the two hemispheres (upper channel) as well as with the two exploring electrodes coupled to a common reference electrode placed on the neck (two lower channels) The simultaneous appearance of slow wave activity in the bipolar and in the two monopolar leads proves that the activity was present at the loci of the cortical electrodes To further control that the bilateral synchrony is real and not a result of the use of the monopolar electrode arrangement as in Fig 28 A, recordings were also made bilaterally with a bipolar configuration as shown in Fig 28 B The distance between the two electrodes on each side was 4—5 mm The fact that the first slow waves seen in the bilateral leads (Fig 28 B, left) are not present in the bipolar transversal lead (upper channel) illustrates the localized character of the activity It is also apparent from the records in Fig 28 that the activity in one side may sometimes lag after the other with a certain delay a phenomenon that will be further dealt with below

From the age of *65—70* days the cortical activity acquires a more differentiated pattern This consists of »waxing and waning regional spindle like bursts with a frequency of 8—14 per sec» (Bernhard *et al* 1967 see also Bernhard *et al* 1959) This type of rhythmic activity which has been called PN I is confined to the parietal region which presumably develops into the middle part of *g suprasylvius* Further studies yielded a more detailed account on the EEG at this age (Meyerson 1967 a and c Bernhard and Meyerson 1968) Fig 29 A and B shows examples of the activity led off with monopolar electrodes from bilateral parietal regions in a 70 days old fetus and it appears that two types of rhythmic activity may constitute the bursts one dominated by 6—8 Hz (left) and one by 14—16 Hz (right) The two types of rhythmic activity often appear in succession In between these composite bursts there is almost complete electrical »silence» The waves of the 6—8 Hz activity tend to be monophasic positive with an arcade shaped form (see Fig 29 B right part) whereas those of the 14—16 Hz bursts are diphasic The high degree of periodicity of both components as well as the similarity in

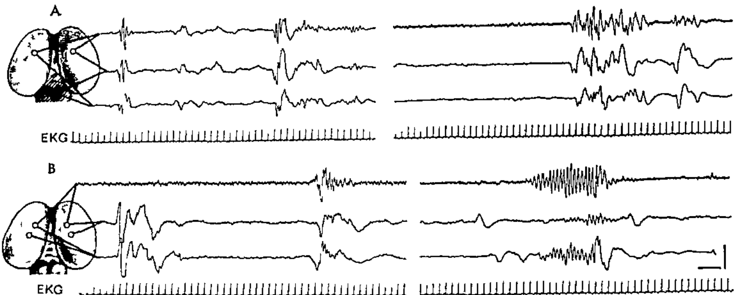


Fig 28 Electrocortical activity in a 60 day fetus A transversal bipolar (upper lead) and bilateral monopolar (lower leads) recordings B transversal bipolar (upper lead) and bilateral bipolar (lower leads) recordings Upper lead in right part of A and in B with horter time constant than in lower leads Calibration 25 μV 1 sec

spectral density pattern between the activities in the two hemispheres are seen in the spectrograms in Fig 29 D (The narrow and sharp peaks at 50 Hz are due to interference from the main power supply)

In most cases the PN I bursts led off bilaterally from symmetrical points appeared simultaneously (Fig 29 A) although the beginning and the end of the epochs were often delayed on either side When the recording was made with a higher paper speed as shown to the right in Fig 29 B the 6—8 Hz waves were seen to be of opposite phase This phenomenon is reflected by the form of the cross correlograms (see Fig 29 C) which display a dip at zero delay and two positive maxima located at almost equal positive and negative delays the values of which correspond to nearly half the period of the dominant correlated component The two correlograms shown in Fig 29 C were computed for two subsequent periods of activity obtained with the same locations of the electrodes and the similarity of the curves illustrates the consistency of the results It should be noted that the correlograms exhibit periodicity even at long delays which indicates a considerable stability of the frequency of the correlated components Cortical activity led off bilaterally from parietal regions in young fetuses of the typical PN I age (70—80 days) almost regularly yielded cross-correlograms displaying a »triphasic» configuration within short delays This result could be confirmed in a large number of recordings and the degree and characteristics of the bilateral synchrony was not influenced by the location of the reference electrode(s)

The confinement of rhythmic activity to the anlage of *g suprasylvius* is apparently quite strict since moving the exploring electrodes laterally from this area to the shallow anlage of *s suprasylvius* unevitably resulted in a marked change of the activity pattern As illustrated in Fig 29 E activity recorded from the frontal and lateral regions displayed a pattern which was polymorphic and primarily consisted of slow wave components none of which accounts for any periodicity comparable to those of the typical PN I Also in these regions the activity was discontinuous with bursts of high amplitude activity and intervening periods of relative electrical »silence» In the medial fringe of the lateral area rhythmic activity may sometimes be seen superimposed on the slow wave activity The spectrograms shown in Fig 29 G are based on activity led off from *s suprasylvius* and reveal that there was a comparatively high energy at lower frequencies and that periodic components were much less pronounced than in activity recorded from more medial positions

The activity led off from the frontal and lateral areas in fetuses 70—80 days old displayed an approximate bilateral synchrony as manifested by the more of less simultaneous occurrence of epochs of high amplitude activity The

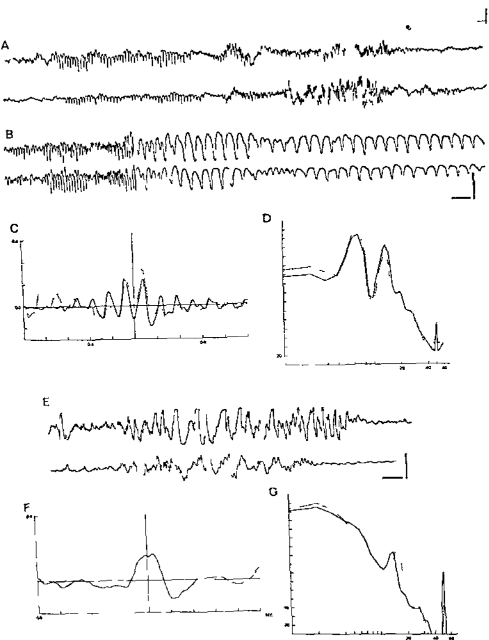


Fig 29 Electrocortical activity in a 70 day fetus A and B bilateral monopolar recordings from the parietal area C cross-correlograms based on two consecutive periods of recording and D spectrograms of bilateral activity illustrated in A and B E activity recorded from the anlage of *s suprasylvius* F cross correlogram and G spectrograms based on activity illustrated in E *Note* right section of B recorded with high paper speed Calibration 50 µV 1 sec B right section 0 25 sec

cross correlograms computed for this type of activity (Fig 29 F) generally had maximal positive values at zero delay and the »triphasic» form as described above was not seen

In fetuses of 70—80 days of age the 6—8 and 14—16 Hz burst activity within the parietal area was sometimes accompanied by slow components The activity in these cases had a discontinuous polymorphic pattern and an example from a 70 days old fetus is shown in Fig 30 It should be noted that although the pattern of each epoch of activity may be different in the two hemispheres they tend to occur with bilateral simultaneity This type of synchrony is relative rather than absolute as being more clearly recognized by the different lengths of the intervening »silent» periods in the two sides It was, however an almost regular finding that during the height of activity in one hemisphere there was also activity to be found in the other



Fig 30 Electrocortical activity in a 70 day fetus Bilateral monopolar recordings from the parietal area Note simultaneous appearance of bursts of activity Calibration 50 μV 2 sec

Bernhard *et al* (1959) noted that the PN I bursts were confined to limited areas of the cortex at a time This observation has been further substantiated in the present study by the finding that cross-correlations for activities led off from nearby points on the *same* hemisphere were comparatively low, although the interelectrode distance was only 3—4 mm Even when the electrodes were placed only 1—2 mm from each other the correlation at zero delay did not exceed + 0 60 (the correlation function was normalized to vary between + 1 and — 1 see Appendix) Cross correlograms with a »triphasic» pattern within short delays were never obtained when activities were led off from one hemisphere only The insular feature of the activity was also apparent in the ink curves and in the example from a 70 day fetus (*g suprasylvius*) given in Fig 31 A a well developed 6—8 Hz spindle could be seen in one of the leads whereas no activity was present in the other (distance between the midpoints of the electrodes was 1 5 mm) The dissimilarity between the two leads as illustrated in Fig 31 A contrasted markedly to the finding that if instead one of the electrodes was placed on a symmetrical point of the *other* hemisphere, an epoch of activity in either lead was almost invariably accompanied by activity also in the other The records in Fig 31 B are from an experiment on an 82 days old fetus (*g suprasylvius*) in which unilateral recordings with an inter

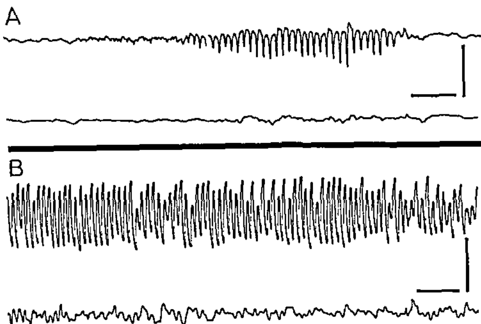


Fig 31 Electrocortical activity monopolar recordings from the same hemisphere A records from a 70 day fetus with separation of recording points 1 5 mm (parietal region) B records from an 85 day fetus with separation of recording points 3 mm (*g suprasylvius*) Calibration 50 μV 1 sec

electrode distance of 3 mm were made Although a characteristic periodic activity of a comparatively high amplitude could be recorded from one of the electrodes almost no activity appeared through the other These findings illustrate that symmetry in the location of the electrodes is a prerequisite for disclosing the presence of bilateral synchrony since there is very little surface spread of the cortical activity

During a fetal age between *75 and 80* days the 6—8 Hz archade shaped activity eventually disappeared altogether and the pattern within the parietal area was generally dominated by high amplitude bursts of 14—16 Hz activity These bursts are often spindle shaped and the frequency of the waves is remarkably constant as revealed both in spectrograms and auto-correlograms (see Fig 32 D and F) Such spindling activity led off bilaterally from *g suprasylvius* in a fetus of about 80 days is shown in Fig 32 A and it is obvious that the spindles appear simultaneously In this case the synchrony was almost perfect in the sense that the spindles begin and end at the same time on the two sides wheras otherwise the activity on one side was often seen to occur slightly ahead of that in the other Bilateral spindling recorded with high paper speed from the same fetus is shown in Fig 32 B and the peaks of arbitrarily

chosen waves of the two records are interconnected with dashed lines in order to illustrate changes of the phase relationship during one and the same burst In this case the waves forming the initial part of the bursts appeared in phase as shown by the vertical orientation of the dotted lines Since the periodic component of the activity in the lower channel in this case apparently had a slightly higher frequency than in the upper the lines interconnecting the wave peaks deviate from the vertical during the last part of the bursts It should be emphasized that when analyzing burst activity in this way the change of the phase angle between the two leads was sometimes seen to occur first in one direction and then back again during the course of one and the same spindling epoch

The cross correlogram shown in Fig 32 C was computed for activity led off from *g suprasylvius* in the same fetus The form of this correlogram is probably caused by the presence of two correlated components of harmonically related frequencies as may also be seen in the auto correlograms in D The two maxima at almost equal positive and negative delay disclose an »opposite phase» relationship between the hemispheres with regard to the component of the lower frequency The low amplitude peak located close to zero delay is presumably a result of the presence of a high frequency component which apparently is bilaterally synchronous with a phase angle of about 0° (a phase relationship of ± 360° is of course equally probable) The graphs in Fig 32 E and F, which are from the same experiment as in Fig 32 C and D illustrate the result obtained when one of the electrodes was instead placed in a heterologous position about 8 mm away from the symmetrical area As shown in Fig 32 E such an asymmetrical location of the focal electrodes resulted in the disappearance of the bilateral synchrony and this effect could not be compensated for by any change of the location of the reference electrode The auto-correlograms in Fig 32 F show the presence of a highly periodic component which is very pronounced in one side but faint in the other a finding that further illustrates the local differences in activity pattern

With increasing fetal age the rhythmic spindle activity becomes less prominent and at *85—95* days it can only be seen in the posterior regions of *g suprasylvius* (Bernhard and Meyerson 1968) In all other areas the activity is continuous and spectral analyses reveal there are generally no dominating periodic components High amplitude burst activity is however present and its appearance seems to be bilaterally synchronous although not as consistently as in younger fetuses The records reproduced in Fig 33 were obtained from a posterior region of *g suprasylvius* (A) and from *g frontalis superior* (C) in a fetus about 85 days old It appears that the pattern displayed in Fig 33 A is highly periodic and similar to that found in somewhat younger fetuses (*cf*

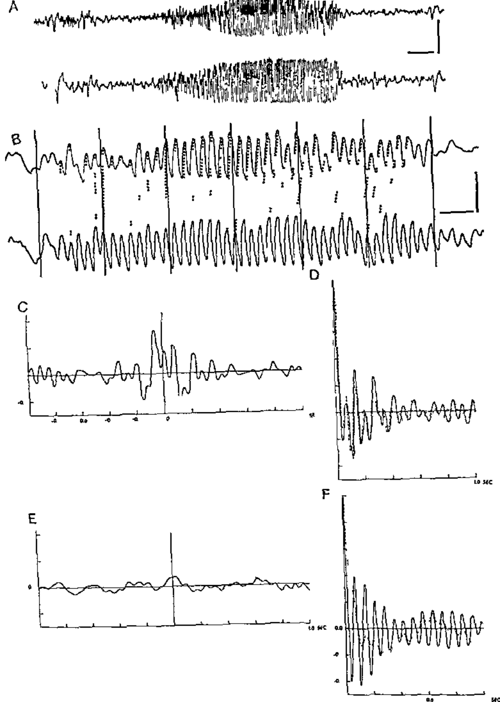


Fig 32 Electrocortical activity in a 80 day fetus A and B bilateral monopolar recordings from g suprasylvius with normal (A) and high (B) paper speed Dotted lines in B interconnect the peaks of arbitrarily chosen waves of the two leads C and D cross and auto correlograms based on activity illustrated in A and B E and F cross and auto-correlograms based on activity of the same types as in A and B but led off from asymmetrical areas Calibration 50 μV 1 sec (A) and 0 25 sec (B)

Fig 32 A) Cross correlation analysis computed for this activity yielded a curve (Fig 33 B) whose form within short delays very much resembled that found in correlograms computed for the same type of activity prevailing at earlier stages of development When, however the activity as in this case was dominated by 14—16 Hz components the cross correlation coefficient at zero delay was variable This variation is presumably due to the fact that even slight changes of the time relation between signals consisting of components with comparatively high frequency will cause considerable changes of the phase angle The uneven distribution of periodicity in the left and right part of the cross correlogram in Fig 33 B reveals that in this case the epochs of correlated activity were relatively short and that they as a rule appeared earlier in the one hemisphere than in the other The section of activity in Fig 33 C which was recorded from *g frontalis superior* has a more polymorphic appearance than that reproduced in A although remnants of PN I activity may occasionally be seen (arrow) A close examination of the activity depicted reveals that there are waves which seem to be bilaterally synchronous a finding confirmed in the cross correlogram in Fig 33 D The pattern of this correlogram should be compared with that obtained for the activity of the PN I type in Fig 33 B

From the age of about *100* days the activity is continuous all over the brain and marked periodic activity of the PN I type is very rare At this age the anterior parts of the brain *i e g frontalis superior* and *g frontalis medialis* may sometimes display epochs of rhythmic 8—10 Hz activity and an example from a 100 days old fetus is given in Fig 34 A The corresponding spectrograms are depicted in Fig 34 B and show high energy at low frequencies and a maximum around 9 Hz Very often the bursts of activity of this type appeared simultaneously in the two sides and the cross-correlation analyses generally disclosed the presence of a certain degree of bilateral synchrony of the individual waves As illustrated in Fig 34 C the cross correlograms computed for activities led off with a monopolar configuration from these anterior regions in this fetus show a »triphasic» configuration a finding which could be reproduced with different arrangements of the reference electrode The pattern displayed by this correlogram illustrates the finding that the correlations obtained were primarily accounted for by periodic components although the content of periodicity in the spectrograms was comparatively low However, when the activity was recorded bilaterally with a bipolar electrode arrangement instead the form of the correlogram changed to a monophasic form (Fig 34 D) In fetuses of this age bilateral synchrony was inconsistently found in the parietal and occipital regions

Towards the *end of the gestation period* the fetal EEG is indistinguishable from that of the lamb when studied under acute experimental conditions The

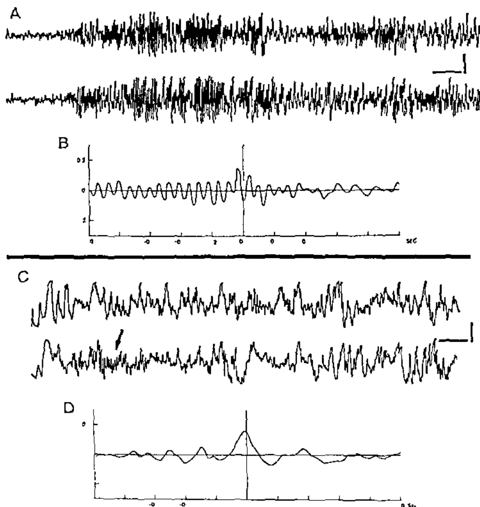


Fig 33 Electrocortical activity in a 85 day fetus Bilateral monopolar recordings from the posterior portion of *g suprasylvius* (A) and from *g frontalis superior* (C) Arrow in C rhythmic activity resembling that of the PN I (see text) B and D *cross correlo* grams based on activity illustrated in A and C respectively Calibration 25 µV 1 sec

wealth of different components of different amplitudes and durations in the cortical activity of these animals easily masks a simultaneous appearance of bilateral events In some cases sudden outbursts of high amplitude waves re minding of the intermittent activity pattern in the immature fetuses may be seen It is noteworthy that such events of activity often occurred simultaneously in the two sides Cross correlation analysis, however, revealed that bilateral wave synchrony was much less consistently present than in fetuses exhibiting

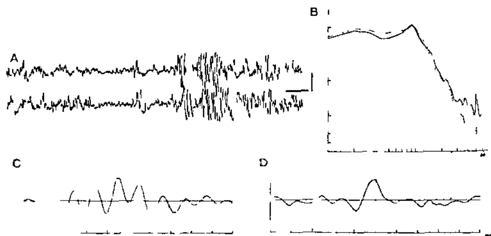


Fig 34 Electrocortical activity in a 98 day fetus A bilateral monopolar recording from *g frontalis superior* B and C spectrograms and cross-correlogram based on activity illustrated in A D cross correlogram based on activity recorded with bilateral bipolar electrodes from the same area as in A Calibration 50 µV 1 sec

rhythmic PN I activity »Triphasic» correlograms were not found Very often the activity in all regions was dominated by sustained high frequency 30—40 Hz activity of low amplitude In those animals—especially lambs and adults —in which this fast activity dominated and which consequently seemed to be in a state of arousal during the experiment bilateral synchrony was rare as judged by cross correlation analyses

In *summary* spontaneous cortical activity is present in fetuses from the age of 60 days When led off from homologous areas the activity displayed bilateral synchrony both in terms of a phase relationship between the individual waves and of a simultaneous appearance of intermittent bursts of activity During the age of 70—80 days burst activity with a highly rhythmic pattern is present within *g suprasylvius* Cross correlations computed for that activity revealed that there was generally an out of phase relationship between the two hemispheres In activity led off from frontal or lateral regions of the brain periodic components were sparse and the cross correlograms showed maximal correlation at about zero delay When the activity was led off from asymmetrical bilateral areas the degree of bilateral synchrony was considerably decreased In fetuses from the age of about 100 days bilateral synchrony was inconsistently found

2 Electrocortical activity after transection of the corpus callosum

With the technique described on p 43 the corpus callosum was transected in five fetuses between 82 and 115 days of age and in one lamb Because of the delicacy of the immature brain operations could not be performed on the youngest fetuses without profuse bleeding and oedema Macroscopical control of brain sections after the experiment showed that the corpus callosum had been totally sectioned in its anterior two thirds The sparing of the posterior third of corpus callosum could hardly be of any importance for the actual problem since comparable callosal lesions have in previous experiments on interhemispheric responses abolished the transcallosal component in all areas which were systematically examined in the present experiments No attempt was made to control the condition of the anterior commissure

Prior to callosal surgery the brain was mapped with bilateral electrodes as described above During the operation the electrodes were left in a position favourable for recording bilateral synchrony

Following transection of the corpus callosum the activity pattern was essentially unchanged although slightly depressed in either or both hemispheres When this depression appeared unilaterally an estimation of the possible persistance of bilateral synchrony as judged from the ink curves was difficult It could be ascertained, however that after callosal section the activity, when led off from regions which in the intact preparation consistently yielded bilateral synchrony generally seemed to retain a certain degree of interhemispheric co ordination This phenomenon was most obvious in records of activity characterized by a more or less discontinuous pattern In these cases the bursts of activity were seen to appear simultaneously in the two sides to a larger extent than would be expected by chance only although the »coupling» between the hemispheres generally seemed to be somewhat more loose than in the intact preparation The records and graphs depicted in Fig 35 were all obtained from an experiment performed on a 95 days fetus Although not of the age when typical PN I activity is found this fetus happened to display rhythmic 14—16 Hz bursts within the middle part of *g suprasylvius* The records shown in Fig 35 were led off with a monopolar electrode configuration from two regions within *g suprasylvius* (A B) after section of the corpus callosum The sections of the records reproduced in the figure were chosen to illustrate the finding that there may be an almost constant co existence of burst activity in the two hemispheres of a callosectomized fetus The cross correlograms depicted in the left row (C E and G) of Fig 35 were obtained from *g ectosylvius frontalis superior* and *suprasylvius* respectively before and those in the right row (D F and H) from the same areas after callosal section It has been stated above that fetuses of the same age as that, from which the records in

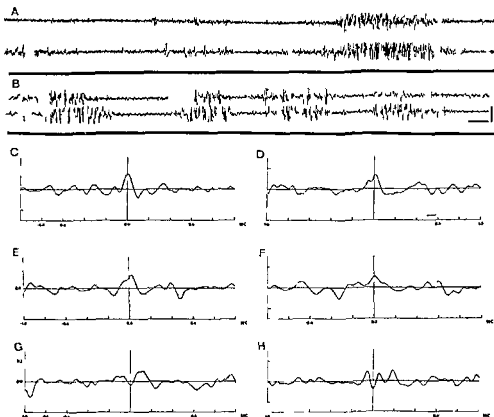


Fig 35 Electrocortical activity in a 95 day fetus after transection of the corpus callosum Bilateral monopolar recordings from the middle (A) and the anterior (B) portions of *g suprasylvius* Cross correlograms based on activity recorded before (C E G) and after (D F H) callosal section in the same fetus C and D *g ectosylvius* E and F *g frontalis superior* G and H *g suprasylvius* Calibration 50 μV 2 sec

Fig 35 have been obtained generally have a low degree of bilateral synchrony as judged from cross correlation analysis Despite of this fact the correlograms obtained in the intact fetus could apparently be reproduced with a similar form after transection of the corpus callosum

It should be noticed that the correlogram obtained for activities recorded from *g suprasylvius* (G) (see ink record in A) has a characteristic »triphasic» pattern which can be recognized also in the graph (H) from the operated animal This regional specificity indicates that the correlations obtained although comparatively low actually reflect a biological entity

In *summary* section of the corpus callosum was performed in fetuses of various ages and did not systematically influence the pattern of the spontaneous

activity After callosal section bilateral synchrony generally persisted both in terms of a simultaneous appearance of bursts of activity and of a phase relationship between the waves as revealed by cross correlation analysis

DISCUSSION

Bilateral synchrony of electrocortical activity

The exploration of the cortical surface for the presence of bilateral synchrony was made by using a monopolar arrangement of the electrodes *i e* in the same way as the mapping of interhemispheric evoked responses In order to exclude the possibility that events occuring at the locus of the common reference electrode might give a falsely high bilateral synchrony the activity was also led off with a bipolar configuration between the two cortical electrodes The fact that, as a rule there was a simultaneity of the activity in the bipolar and in either of or in both the monopolar leads proves that the activity is actually led off by the exploring electrodes and not the common reference

Another factor which might give a seemingly high degree of bilateral synchrony is the spread of current across the midline However with the cortical electrodes used the activity appears to be picked up from a comparatively discrete area, since the cross-correlations computed for activities led off from two nearby cortical points on the *same* side were always comparatively low even with an interelectrode distance of only a few mm The further fact that the form of the cross-correlograms may change with different positions of the exploring electrodes along an anterior posterior plane—keeping the interelectrode distance across the midline constant—indicates that the spread of current is of minor importance In addition the bursts of the discontinuous fetal activity very often appeared with a variable delay in either hemisphere a finding which contradicts any significant artefactual influence both of spread of current and of »activity» led off by the common reference

In an earlier study it was found that when recordings were made with monopolar electrodes directly from the exposed cortex in adult animals there was no systematic relationship between the results of cross correlation analyses and the location of the reference electrode(s) (Meyerson and Moller in preparation) These experiments also showed that in *adult* animals (sheep cat and monkey) bilateral synchrony of cortical activity was very inconsistently present under acute experimental conditions Because of the different geometry of the fetal skull and brain compared to that of an adult animal and of the different impedances in immature and mature brain tissue it was considered

pertinent to record also the fetal cortical activity with various arrangements of the electrodes A large number of such recordings was made but the results of cross correlation analyses were not significantly influenced by the location of the reference electrode(s) (see Methods p 25)

Thus it may be concluded that in the current study the presence of bilateral synchrony as revealed by the ink records or by the cross correlograms does reflect a simultaneous occurrence of cortical events on the two sides

It is a characteristic feature of the earliest spontaneous activity in the fetal sheep that it is generally bilaterally synchronous as manifested both by a phase relationship of the slow wave components and by a simultaneous appearance of intermittent burst activity During the developmental stage which is characterized by the presence of PN I activity, *i e* at an age of 70—80 days, bilateral synchrony is particularly well pronounced The occurrence at this age of 14—16 Hz biphasic waves with a very consistent form and frequency is notable since it suggests the functioning of highly organized circuit mechanisms in a brain that is still comparatively immature This type of activity however, resembles that of other species during early stages of development (see Bernhard and Meyerson 1968) although it seems to be particularly prominent in the sheep It is worthy of note that the characteristic 6—8 Hz arcade shaped waves often found during a short period of ontogenesis in sheep may occasionally also be observed in the newborn child (Dumermuth personal communication)

Data on the development of bilateral synchrony of spontaneous cortical activity are not found in the literature However the problem has been commented upon in studies performed with scalp electrodes and confined to the postnatal period Thus Caveness (1962) in an extensive investigation on the EEG development in the monkey demonstrated that spindling activity concomitant with states of drowsiness and light sleep is present from birth and that such spindles often appear bilaterally synchronous in homologous areas Bilateral synchrony of this activity was found to be most pronounced during the first months of life and seemed to diminish thereafter The activity displayed during light sleep in the monkey consists of spindles of 15—18 Hz and is thus similar to that described as PN I in the fetal sheep Likewise, in both species such activity tends to become rare with increasing age

In the developing dog Fox (1967) found »neo-natal spindles» of 10—18 Hz to be present during the first weeks of life and these spindles displayed bilateral synchrony Similar spindle activity has also been demonstrated in the premature baby during the period between 24 and 28 weeks conceptional age (Dreyfus Brisac 1962) and these spindles tend to appear bilaterally synchronous (Dreyfus Brisac personal communication) Bilateral synchrony of other

types of activity in man does however, not become apparent until the time of birth (Dreyfus Brisac 1962 1964)

As noticed by Bernhard *et al* (1959) the PN I spindles appear in limited areas at a time and this observation has been fully confirmed in the present investigation (see also Verley 1967 b) It was furthermore found that the spindle bursts appear bilaterally and in restricted symmetrical areas

In a study on the functional significance of thalamo cortical connections for the appearance of barbiturate spindling Andersen *et al* (1967) presented experimental evidence for a strict point to point thalamo cortical relationship It was assumed that there is a columnar thalamo cortical organization the cortical extent of which does not exceed 2 mm and which accounts for the localized character of the spindles Bilateral synchronous appearance of barbiturate spindles was found to be rare in that study It should be pointed out however, that the sensory motor and not the association areas were primarily examined Barbiturate spindles have many features in common with spindling activity seen in light sleep (see *e g* Ralston and Ajmone Marsan 1956) and in the adult animal the appearance of spindles in light sleep as well as during barbiturate narcosis has generally been found to be bilaterally synchronous (*e g* Swank 1949 Rossi *et al* 1963) It is not unlikely that the insular appearance of PN I spindles may be due to a certain degree of columnar organization similar to that demonstrated for barbiturate spindles which would imply that in essence point to-point thalamo cortical connections are functionally mature at an early stage of development

The thalamic driving of the bilateral spindling has been pointed out by *e g* Milhorat *et al* (1966) and has been regarded to be a release phenomenon due to the cessation of influences emerging from brain stem activation centers (Rossi *et al* 1963) The spindle activity displayed within the parietal region in fetuses between 70—80 days of age may not be modified by external stimuli (*cf* Bernhard *et al* 1959) and the uniform spindling feature of the activity might be due to a lack of activating inflow from the brain stem during this developmental phase

Cross correlagrams computed for the arcade shaped 6—8 Hz activity as a rule displayed a »triphasic» pattern *i e* with a low or even negative value at zero delay and two positive maxima located at almost equal positive and negative delays Correlograms with such a feature of activity led off bilaterally from the exposed cortex have not earlier been reported Since it is highly unlikely that the occurrence of »triphasic» correlograms is the result of the method of recording as well as of spread of current, it is assumed that such correlograms obtained from young fetuses do reflect a physiological entity It seems reasonable then to interpret the »triphasic» cross correlograms as reflecting a

state in which the activity in either hemisphere leads or lags. The magnitude of the positive and negative delays to the peak of each of the positive maxima denotes the deviation from zero degree phase relation. The low correlation at zero delay further reveals that an in phase relation between the individual waves of the two sides was in these cases relatively rare. Cross correlograms with a consistent monophasic appearance, i.e. a positive maximum located at zero delay which is sometimes surrounded by minor peaks at increasing positive and negative delays, were found when processing activity other than the rhythmic PN I. These results substantiate the view that there is a functional coupling between the two hemispheres, a conclusion further supported by the finding that activity dominated by 14—16 Hz burst activity (see Fig. 30 A and 31 B) generally yielded substantial cross correlation coefficients, although the values at zero delay were variable.

The firm functional linkage between the two hemispheres as manifested by a consistent bilateral synchrony present already at 70—75 days of age is unexpected and contrasts with the considerable functional and structural immaturity otherwise prevailing in this phase of brain development (see Bernhard *et al.* 1967, *cf.* Kolmodin and Meyerson 1966). It would therefore be tempting to assume simple callosal mechanisms to be responsible. However in the immature sheep brain the corpus callosum is apparently not crucial in this respect since bilateral synchrony may persist after callosal section. This conclusion is substantiated by further experimental evidence. Thus, the fact that the cross correlograms may display a maximal positive correlation located at zero delay is difficult to account for by callosal mechanisms. The correlograms were computed with a resolution of ± 5 or 10 msec and since the minimal time of transcallosal transmission as estimated by the onset latency of TCR in the actual fetal age amounts to 40—50 msec (see page 54), an in phase relation could hardly be explained by callosal functions. Furthermore, the spindle activity (PN I) which almost regularly displayed bilateral synchrony is strictly confined to the middle part of *g. suprasylvius*, an area which seems to be poorly supplied by callosal fibers as reflected in the inconsistency of TCR s elicited within that region. Instead, the predominance of IDR s within this area in the fetal sheep indicates the existence of more elaborate extracallosal than callosal interhemispheric connections. One might assume therefore that the bilateral synchrony of activity which is confined to *g. suprasylvius* is largely dependent upon deep-seated mechanisms which may involve brain stem structures serving also the transmission of the IDR. This hypothesis seems to agree with the statement of Bremer (1966 c) that »the reticular formation with the thalamic nonspecific system may act as a long loop commissural link and a synergy mechanism between the two cerebral cortices». On the other

hand it is not necessary to assume the participation of brain stem structures which, together with the thalami would constitute a fairly complex system With the supposition that thalamic mechanisms are engaged in the production of cortical spindle bursts, commissural fibers within the massa intermedia might account for the bilateral synchrony Though inconsistently present in man and primates this structure is well developed in lower mammals (*cf* Glees and Wall 1948) The importance of the massa intermedia as an interhemispheric link for bilateral appearance of evoked cortical events has been demonstrated by Enomoto (1959) and Bava *et al* (1966) Furthermore, according to Heuser *et al* (1961) so called caudate spindles the pattern of which is very similar to other types of spindling activity, generally display bilateral synchrony which is maintained by extra callosal pathways partly via thalamic structures

In conclusion, there is much evidence in favour of the view that bilateral synchrony of spontaneous cortical activity is present in the sheep at an early stage of functional development and moreover, that this synchrony is primarily dependant upon the functioning of extra callosal mechanisms

The inconsistency of bilateral synchrony in fetuses near term as well as in the adult animal as compared to the young fetuses may be due to different factors In this context one has to consider the fact that the cortical activity of the immature brain is very uniform in comparison to that recorded in the fully developed animal in which the EEG may display a great variety of patterns reflecting different levels of wakefulness and sleep This implies that the activity of the mature brain is almost continuously modified by the impact of afferent signals which may easily mask or distort the presence of bilateral synchrony on a cortical level In the adult animal (cat) it has been found that cross correlations obtained for bilateral cortical activity were highly variable even when computed for consecutive periods of recording and with the electrodes in the same position (Meyerson and Moller in preparation)

As outlined in the introduction there is no conclusive evidence as to the role of the corpus callosum for the appearance of bilateral synchrony in adult animals In studies on normal and pathological spontaneous activity in man Aird and Garoutte (1958) adhere to the view that a central »cerebral pace maker» is almost solely responsible for bilateral synchrony whereas the commissural system is not »essential» Bilateral contemporarity of bursts of activity was particularly noticed and was ascribed to the function of »some common midline paramesial sources» possibly engaging *n reticularis thalami* (Hasegawa and Aird 1963 see also Ralston and Ajmone Marsan 1956) With the use of non automatic methods of analysis it was shown that there is a tendency of the EEG waves in each hemisphere to lead alternately in time resulting in a shifting asynchrony presumably due to a sporadic »escape» of cortical

activity from deep seated pacemaker mechanisms (Ogden *et al* 1956 Garoutte and Aird 1958) The finding of a continuous change in time of the bilateral synchrony has been confirmed in the present investigation by the observation that the interhemispheric phase angle may change slightly in both directions during one and the same burst of 14—16 Hz activity

Opposing the concept of central pacemaker Bremer (1966 a see also Bremer 1956, Bremer and Stoupel 1957) and Berlucchi (1967) claim that the callosal system is of crucial importance for bilateral synchrony Such a definite conclusion does however not seem wholly justified in view of experimental findings made by Radulovcki *et al* (1965) and Batini *et al* (1967) The lack of quantitative experimental data such as yielded by cross correlation analysis is a serious drawback for the final solution of the problem In the current study it has been shown that in the immature brain callosal mechanisms are not necessary for bilateral synchrony whereas the data obtained from adult animals have apparently been contradictory One possible explanation for the different results obtained may be that in the young fetus extra callosal connections are relatively more significant than callosal mechanisms which become more important for bilateral synchrony with advancing development

SUMMARY

The development of interhemispheric functional relations has been investigated with electrophysiological methods Two different electrocortical signs of inter hemispheric functions have been chosen 1) interhemispheric responses (IHR) which refer to the whole sequence of gross potentials led off from the cortical surface in response to electrical stimulation of a homologous area in the other hemisphere The response comprises the »transcallosal response» (TCR), me diated via the corpus callosum, and the »interhemispheric delayed response» (IDR) which is transmitted in extra callosal pathways 2) bilateral synchrony of spontaneous normal cortical activity with special regard to the significance of callosal and extra callosal mechanisms

Acute experiments were performed on unanesthetized and externalized sheep fetuses kept in umbilical contact with the decerebrated ewe on lambs and on adult animal Fetuses of 60 days of age to full term (145 days) were used

1 In all the sheep except in the very youngest fetuses examined the IHR consisted of an initial complex of potentials, generally positive negative and of late components of variable form and latency Transection of the corpus callosum as well as stimulation of and recording from this structure in the intact preparation (both pre and postnatally) proved that the initial com plex is equivalent to a TCR and the late components to an IDR. In the *adult sheep* TCR s were most consistently obtained in the primary sensor and motor areas whereas in associations areas (anterior and middle parts of *g supra sylvius*) responses appeared irregularly IDR s were less consistently found in the adult than in the immature sheep unless facilitated by *a* chloralose in which case they were present in most areas

Cortical responses to contralateral stimulation could first be elicited in *68 —70 days old fetuses* within a limited fronto parietal area which eventually develops into *g suprasylvius* These responses are mediated via the callosal system From the same age it is also possible to evoke a direct cortical response (DCR) indicating that at this stage of development the cortex has become excitable to electrical surface stimulation Since DCR s were not confined to any particular area the appearance during the following development of TCR s in regions outside that in which they were first found is primarily de

pendent upon the successive outgrowth of callosal fibers

During the first week of its presence the TCR generally consisted of a monophasic positive deflection of long latency and low amplitude The response was unable to follow repetitive stimulation of frequencies above one per 5—15 seconds The threshold was high and the form of the response was not influenced by the stimulus voltage The functional background of the positive surface response has been discussed on the basis of data on the development of the somesthetic response in the fetal sheep and on the morphology of the developing cortex The surface positive deflection of the TCR has been ascribed to either a depolarization of callosal terminals—prior to the establishment of synaptic contact with cortical neurons—or to excitatory postsynaptic activation in deep cortical strata Hyperpolarization processes in superficial layers of the cortex have been considered unlikely

Around the *80th day* of fetal life the positive deflection of the TCR was *consistently* followed by a negative potential which caused *the response pattern* to resemble that of the mature animal The negative phase of the TCR presumably represents postsynaptic cortical events as indicated by its relative inability to respond consistently with repetitive stimulation

The onset latency of the TCR was at the 80th day *longer* than that found at the time of the earliest appearance of the response (70 days) This implies that the increase of the transcallosal cortico-cortical distance which takes place between the 70th and 80th day is not sufficiently compensated for by a simultaneous increase of the maximal conduction velocity Stainable myelin was not present in the callosal system at this stage of development (Loyez stain)

Although TCR s were first observed within the anlage of *g suprasylvius* the responses evoked from this area became irregular from the age of 80—90 days Instead responses with a consistent form and high amplitude were most easily obtained from primary sensory and motor areas Thus from this stage of development the distribution of TCR s was similar to that found in the adult sheep

From the age of *90—100 days* the TCR evoked by suprathreshold stimulation was followed by IDR components of variable configurations Within *g suprasylvius* IDR s were very prominent and even appeared alone without any sign of a TCR In primary sensory and motor areas both responses were generally equally well pronounced The IDR failed to follow all but the lowest frequencies of stimulation (0 2—0 5 Hz) and was blocked by asphyxia within a short time

The later appearance in the development of an IDR than of a TCR is indicative of a delayed functional maturation of extra callosal as compared to

callosal interhemispheric connections in ontogeny

During a developmental period between approximately 90 and 110 days both the TCR and the IDR, as a rule, appeared with a relatively constant form and with a higher amplitude than was found in either earlier or later stages This phenomenon has tentatively been ascribed to the lack of inhibitory influences exerted from lower centers

From the age of about 80 days the onset latency of the TCR slowly decreased and at the time of birth it approximated half the value found in the adult sheep On the basis of the onset latency and transcallosal cortico-cortical distance, measured on coronal brain sections the approximate maximal conduction velocity of the callosal fibers was estimated During almost the whole prenatal period the maximal callosal conduction velocity was low although there was a slight but consistent increase from about 0 5 to 1 m/sec Since there is no stainable myelin present during this same period the change of conduction velocity is presumably due to the increase of the diameter of the still unmyelinated callosal fibers Towards the end of the gestation period a marked increase of the conduction velocity could be observed until about 40 days postnatal age when values comparable to those found in the adult animal were attained This last phase of development is characterized by the formation of myelin in the callosal commissural system

2 Bilateral synchrony of spontaneous cortical activity in the developing sheep has been studied with reference to simultaneous bilateral appearance of epochs of activity and to phase relationship between the individual waves of activity led off bilaterally Cross and auto-correlation analyses as well as power spectral analyses were performed with the use of a general purpose digital computer

Spontaneous cortical activity is present in the fetal sheep from the age of about *60 days* The activity is discontinuous and consists of slow waves appearing isolated or clustered between which there is electrical »silence» Both the isolated slow waves and short epochs of activity generally appeared bilaterally synchronous

Between *70 and 90 days* of fetal age the activity prevailing in parietal regions is characterized by the presence of bursts which often appear with a spindle form and consist of rhythmic activity with a comparatively stable frequency Such bursts were only present within small circumscribed areas of the cortex at a time and the cross correlation coefficients computed for activity led off from nearby points on the same hemisphere were comparatively low The insular appearance of the rhythmic bursts as well as the characteristics of the activity pattern suggest that already in this phase of development functional thalamo-cortical connetions have been established to some extent As a rule rhythmic burst activity appeared simultaneously in homologous areas and

there was a phase relation between the individual waves Cross-correlograms computed for this type of activity showed that the waves in the two hemispheres appeared in synchrony but with a delay in either side Bilateral recordings from heterologous areas resulted in a considerable decrease of the synchrony

From about *90 days* of fetal age, cortical activity is continuous and displays a more polymorphic pattern than in earlier stages of development Rhythmic activity is rarely present In fetuses of this age group as well as in lambs and adult animals bilateral synchrony was inconsistently present

In several fetuses of ages between 80 and 115 days the corpus callosum was transected and it was found that the bilateral synchrony was preserved to a considerable extent as shown both by a co-existence of epochs of activity in the two hemispheres and by a phase relationship as disclosed in the cross correlograms This finding led to the conclusion that at least in the immature brain the *callosal system is not crucial for the* maintenance of bilateral synchrony of spontaneous cortical activity This view is further supported by the fact that bilateral synchrony is particularly well pronounced in activity led off from *g suprasylvius* which area seems to be poorly supplied by callosal fibers as judged from the inconsistency and irregular appearance of TCR s

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where the sequence X_1, X_2, X_3, ... X_M is the amplitude values of the signal at time Δt, $2\Delta t$, $3\Delta t$, ... $M\Delta t$, Δt being the sampling interval. The auto-correlation ac_j is computed as a function of j which represents the number of Δt with which the signal is delayed.

The cross correlograms were computed in a similar way,

$$cc_j = \frac{\sum_{k=1}^{M} X_k \cdot Y_{k+j}}{\sqrt{\sum_{k=1}^{M} X_k^2 \cdot \sum_{k=1}^{M} Y_k^2}} \qquad \text{Eq. 2}$$

where the sequences X_1, X_2, X_3, ... X_M and Y_1, Y_2, Y_3, ... Y_M are the amplitudes of the two recorded signals at times Δt, $2\Delta t$, $3\Delta t$, ... $M\Delta t$. The cross correlation was computed as a function of j which takes positive as well as negative values.

The numerator of Eq. 1 and 2 has generally been used alone to express the correlation. In order to compare quantitatively the cross correlations of data obtained in different experiments in which the amplitude of the activity was likely to vary, the auto- and cross-correlations were normalized by the denominator as shown in Eq. 1 and 2. Computed in that way the values of the auto- and cross correlation functions range between — 1 and + 1.

The auto correlation function was computed to a maximum lag of 530 or 1060 msec in intervals of 5.3 or 10.6 msec. The cross correlation function was computed with the same intervals between delays of ± 530 or 1060 msec.

The power spectrum of both channels was computed by Fourier transformation of the computed auto-correlation functions and the outcome was smoothed by hanning (Blackman and Tukey 1958). First the raw spectral density estimate was obtained:

$$P_r = ac_0 + 2\sum_{j=1}^{m-1} ac_j \cos(2\pi r \Delta f\, j \Delta t) + ac_m \cos(2\pi r \Delta f\, m \Delta t) \qquad \text{Eq. 3}$$

for $r = 0, 1, 2, 3, \ldots p$. The frequency f is $\Delta f \cdot r$ where $\Delta f = \frac{1}{2m\Delta t}$,

$m\Delta t$ being the maximal delay of the auto-correlation. From the raw spectral density estimates P_r the refined spectral density estimates P' are computed by smoothing (hanning) according to

$$P'_r = 0.23\, P_{r-1} + 0.54\, P_r + 0.23\, P_{r+1} \qquad \text{Eq. 4}$$

The power spectrum was computed in the frequency range 1 to 80 Hz An increase of the resolution results in a decrease of the stability of the spectral estimates with the analysis time kept constant With regard to the purpose of the present investigation the resolution of 1 Hz was considered sufficient

Since the normalized auto-correlograms were used as a basis for power spectrum computation, the absolute value of the obtained spectrum estimates bears no relationship to the absolute amplitude of the recorded electrical signal Normally the absolute value is of no interest but it can easily be introduced by incorporating the mean square value of the signal which was computed for the purpose of normalization of the auto correlogram (the denominator in Eq 1)

As mentioned by Blackman and Tukey (1958), there is a univocal relation between the sample length and spectral resolution on one hand and the stability of the power spectrum estimate for stationary signals on the other With the sample length and the resolution given the accuracy can be predicted Because of the nonstationary character of the normal EEG signal such predictions cannot be relied upon In this connection it should be mentioned that Weiss (1959) has demonstrated that the auto correlation of short samples at long delays shows peculiarities that are not referable to the properties of the signal We therefore made an empirical evaluation of the required sample length and it was judged that a sample length of 50 seconds yielded data of sufficient stability for the purpose of the investigation

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Studies on the Regional Renal Blood Flow with P^{32}-Labelled Red Cells and Small Beta-Sensitive Semiconductor Detectors

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Studies on the Regional Renal Blood Flow with P^{3} -Labelled Red Cells and Small Beta-Sensitive Semiconductor Detectors

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Contents

CHAPTER I

Purpose of the investigation

The intrarenal blood flow distribution began to receive serious attention with the introduction of the counter current theory for the urine concentration mechanism (Wirz, Hargitay and Kuhn 1951 Hargitay and Kuhn 1951) At this time quantitative data were available of the size and regulation of the total renal blood flow (Smith 1951) Less interest had been paid to the medullary circulation with the exception of the blood flow distribution under the influence of vasoactive substances renal nerve stimulation and haemorrhagic shock (Trueta et al 1947) What now focused interest on the blood flow distribution was the belief that the medullary circulatory system was directly involved in the process of excretion of concentrated and of dilute urine essential to the water balance Problems connected with this assumption have led during recent years to a number of investigations of the magnitude and regulation of the blood flow in different parts of the kidney

Several methods have been developed for the determination of regional renal blood flow The method most extensively employed is the PAH (para aminohippuric acid) extraction method (Reubi 1958 1961) This method is based on the assumption that blood perfusing the cortical parenchyma is completely cleared of PAH whilst no PAH is extracted from the blood perfusing the medulla There has been no acceptable verification of this basal postulate however and values obtained by the method have therefore to be interpreted with caution Inert gas methods (Thorburn et al 1963 Aukland et al 1964 Aukland and Berliner 1964 Aukland and Wolgast 1968) to which group the heat dilution methods (e g Schieve et al 1959 Sadler and Tuttle 1963, Scher 1951 Perl and Hirsch 1966 Grangsjo et al 1966a) can also be assigned are found to be unsuitable for the determination of medullary blood flow since highly diffusible indicators will be trapped in the counter current exchange system formed by the ascending and descending vasa recta As regards the measurements in the inner medulla the urine flow will influence the wash out rate These drawbacks are probably also inherent in the Rb^{86} accumulation method (Harsing and Pelley 1965) in which the amount of Rb^{86} accumulated in a region in one minute is used as an index of regional blood flow These methods will nevertheless give useful information on the transport capacity of the medullary circulatory system The term nutrient or effective has been applied to characterize the blood flows obtained by these methods

Only two methods have been developed which permit the determination of absolute (in contrast to effective or nutrient) regional renal blood flow viz the method of Kramer et al (1960) and that of Lilienfield and Maganzini (1960)

In the latter method the accumulation rate of I^{131} Labelled albumin was used as an index of regional blood flow The main drawback of this method is that it requires

a whole series of animals to obtain *one* accumulation curve and thus *one* blood flow determination

In the photoelectric method of Kramer and coworkers the regional blood flow was calculated from the *regional blood volume* and the *mean transit time* for a protein bound dye in the different regions of the kidney The regional blood volume was in turn obtained from direct measurements of the regional red cell volume and from published data of intravascular haematocrit The mean transit times in the cortex and medulla were calculated from dye dilution curves obtained from these regions after a slug injection of Evans blue or Cardiogreen into the renal artery The cortical recordings were taken from a photoelectric reflectometer pierced under the capsule The medullary recordings were made by means of an arrangement consisting of a lamp inserted into the middle of the medulla and a photoelectric probe inserted into the pelvis and placed along one side of the medulla (inner zone) In a later publication (Deetjen et al 1964) a modification of the method was presented which permitted blood flow determination in the outer zone of the medulla The main drawback of this method is that it is technically complicated and that the regional blood volume or changes in regional blood volume cannot be measured directly In a wider sense it has in addition the limitation that only dyes can be used as labelling substances

It is thus obvious that although several methods have been developed for determination of the regional renal blood flow none of them is entirely satisfactory

In this work a new *indicator dilution method* is presented with P^{32} *labelled red cells as the indicator* the radioactivity being recorded by *small needle shaped semiconductor detectors* inserted into the parenchyma

The purpose of the work can be summarized as follows

1 To investigate the properties of the recording equipment
2 To investigate the possibility of determining the mean transit time for an intravascular indicator by internal monitoring with these needle detectors
3 To study the blood flow in the different regions of normally functioning kidneys
4 To study the cortical and medullary blood flow as related to the perfusion pressure and to osmotic diuresis induced by mannitol

The experiments were carried out on dogs

CHAPTER 2

Anatomy and physiology of the renal vascular system

A Anatomy[1]

In the mammalian kidney the renal artery divides into the interlobar arteries radiating from the hilus and passing along the pyramids before penetrating the renal parenchyma at the level of the cortico medullary border Here they give rise to several branches the arcuate arteries which pass as arches along the cortico medullary border (Fig 1) From these arteries arise the interlobular arteries which extend into the cortical parenchyma On their way through the cortex they give off at more or less acute angles, the afferent glomerular arterioles In man and in the dog and cat these arteries finally pierce the capsule where they end in a number of branches anastomosing with extrarenal arteries The afferent glomerular arteriole breaks up into the glomerular capillary system, the vessels of which reunite to form the efferent glomerular arteriole The efferent arteriole of the cortical glomeruli then breaks up into the peritubular capillaries supplying the cortical tubuli and collecting ducts These capillaries then empty into the interlobular veins accompanying the interlobular arteries In some animals including the dog and man there is in addition a superficial venous drainage The interlobular veins then drain into the arcuate veins joining to form the interlobar veins which in turn join to form the renal vein

The medullary parenchyma derives its blood supply from the juxtamedullary glomeruli located in the inner part of the cortical parenchyma The number of these glomeruli averages according to Edwards (1956) 18 % of the total number of glomeruli which in the human kidney is about 1 000 000 The efferent arteriole of the juxta medullary glomerulus is described by Fourman and Moffat (1964) as a stout short trunk dividing into a variable number (up to about thirty) of vasa recta In addition to the vasa recta several small branches are given off to supply the subcortical zone Some of these branches may arise from the proximal part of the vasa recta The descending vasa recta extend into the medullary parenchyma as bundles in close contact with the corresponding ascending vasa recta This spatial arrangement of the vessels is most pronounced in the outer medullary zone whereas in the inner zone the vessels are more evenly distributed These descending vasa recta end at different levels in the medulla in capillary systems (Fourman and Moffat 1964 Hakke and Pfeiffer 1964) which in the outer zone form a dense capillary network intermingled between the vasa recta bundles and in the inner zone a less dense network made up of elongated meshes Many of the descending vasa recta extend unbranched to the papillary plexus The medullary capillaries then join to form the ascending vasa recta emptying into the arcuate veins An additional blood supply

[1] For more detailed descriptions reference may be made to von Mollendorff and Schroder (1930) and Fourman and Moffat (1964)

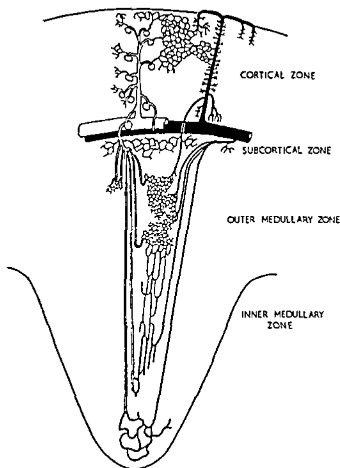


Fig. 1. Diagrammatic representation of the vascular bed in the kidney. Note the dense capillary network in the outer zone of the medulla and the less dense network made up of elongated meshes in the inner zone. (From Fourman and Moffat, 1964, by permission.)

to the papillary region is derived from vessels located in the adventitia of the minor calyces.

In a recent electronmicroscopical study by Moffat (1967) it was found that the efferent arterioles of the juxtamedullary glomeruli and the proximal part of the vasa recta include a layer of smooth muscle cells. The innervation was thought to be cholinergic in nature in contrast to the smooth muscle coats of the purely cortical vessels which are accompanied by adrenergic nerve fibers. In the inner strip of the medullary outer zone the muscle layer is replaced by perivascular cells containing myofibrils and located between the descending and ascending vasa recta. In the inner zone these cells were found to be successively replaced by medullary interstitial cells not containing any contractile elements.

The endothelium of the descending vasa recta is relatively thick, whilst that of the ascending vasa recta is thin and fenestrated (Longley et al. 1960; Moffat 1967).

The vascular volume is essentially the same within the different regions of the kidney, and in dogs according to Lilienfield et al. (1958) and Emery et al. (1959) it amounts to 30 % or more of the tissue volume. (The value given probably repre

sents an overestimation of the vascular volume since in these studies I^{131} labelled albumin, which will be partly located extravascularly was used to determine the plasma volume)

The blood volume distribution between main arteries and veins was investigated by Weaver et al (1956), using a method involving injection at physiological pressures of liquid latex into the vascular system fixation of the rubber and then measurement of the volume of the rubber casts The volume of arteries below the cortex (including the arcuate arteries) was found to be essentially the same as that of the corresponding veins (2 4 % and 2 6 % of the distended kidney volume respectively) The total volume of main arteries and veins (including the interlobular arteries and corresponding veins) was found to be 4 5 % and 7 % respectively The volume of descending vasa recta is as mentioned by Thurau in a review (1963) less than that of the ascending limbs, which is consistent with the higher pressure drop in the descending vessels

B Functional implications

From the description of the renal vascular arrangement it stands clear that there are two main vascular systems in the kidney the one made up of the cortical glomeruli and the peritubular capillaries and the other of the juxtamedullary glomeruli and the vasa recta system Both these systems are intimately related to the excretory function of the kidney — the cortical system to the glomerular filtration and to the tubular reabsorption and excretory processes and the juxtamedullary system to the process of urine concentration and dilution The latter process will be considered in some detail here

It is now generally accepted (Berliner et al 1958 Smith 1959 Lamdin 1959 Ullrich 1959 Ullrich et al 1961a Giebisch 1962 Berliner and Bennett 1967) that the driving force of the urine concentration process is the outward transport of sodium in the ascending limb of the loop of Henle which is assumed to be impermeable or at least only slightly permeable to water The urine delivered to the distal convoluted tubuli would therefore be hypotonic with respect to plasma Due to the counter current system formed by the descending and ascending limbs of the loop of Henle a successively increasing concentration of solutes is created in the direction towards the papilla primarily consisting of sodium chloride and urea In antidiuresis the hypotonic urine created in the ascending limbs becomes equilibrated with plasma in the distal convoluted tubuli which are water permeable in the presence of ADH (antidiuretic hormone) The final concentration of the urine is achieved as it flows through the medullary collecting ducts by an outward movement of water into the hypertonic medullary interstitium During water diuresis the water permeability of the distal tubuli and collecting ducts is reduced The urine traversing the ascending limbs of the loops of Henle and distal convoluted tubuli will become hypotonic by the outward transport of sodium in these segments and will remain so as it flows through the water impermeable (or slightly water permeable) collecting ducts

The vasa recta which also form a counter current system are generally held to act as a passive counter current exchange system Such systems have been described previously for the maintenance of high oxygen tensions in the swim bladder of deep sea fish (Scholander 1954) and for the conservation of heat in the sloth (Scholander and Krog 1957) The functional significance of the vasa recta counter current exchange system in the kidney is that it creates a barrier to the net transport of diffusible substances along the long axis of the vasa recta limbs Solute removed from the hyperosmotic medullary interstitium by ascending vasa recta blood, which thus gains a high solute concentration will be passively transported to the descending vasa recta which have a low solute concentration The amount of solute shunted in this way is dependent upon the existing concentration difference the spatial arrangement of the vasa recta and the diffusion characteristics of the solute in question The amount carried away by the blood is proportional to the concentration difference and the blood flow Water as well as substances (e g oxygen) carried by the arterial blood to the medullary regions will be shunted in the opposite direction This problem has been treated theoretically by several investigators (Scholander 1954, Berliner et al 1958 Ullrich et al 1961a Gunzler in Thurau and Deetjen 1962) A more general theoretical treatment was given by Niesel and Röskenbleck (1963) who pointed out that the efficiency of a counter current exchange system is greatly reduced when the outflow exceeds the inflow in the system

There is in fact experimental evidence of a highly effective counter current exchange system in the kidney Micropuncture studies of Wirz (1953) Gottschalk and Mylle (1959) and Ullrich et al (1961 b) have shown that the osmolarity of vasa recta blood is essentially the same[1] as that of the fluid in the tubuli and collecting ducts at the same level in the medulla The slow uptake of diffusible substances such as sodium water and krypton (Morel et al 1960 White et al 1961 Lassen and Longley 1961) as well as the low inner medullary and urinary oxygen tension (e g Rennie et al 1958 Aukland and Krog 1961 Aukland 1962, Ulfendahl 1962 a Aperia and Liebow 1964) also support this finding

An alternative hypothesis that the vasa recta system acts as a counter current multiplier the driving force being the hydrostatic pressure difference between the vasa recta limbs has been outlined by Lever (1965) This hypothesis has been criticized recently by Berliner and Bennet (1967) who emphasize the improbability that the hydrostatic pressure is able to create the high solute concentrations found in the medulla

Pinter and Shohet Pinter and Shohet 1963 Shohet and Pinter 1964) have outlined a theory in which the high solute concentrations in the inner zone could be created by the vasa recta system (or by the loops of Henle) when the counter-current multiplier is limited to the thick ascending part of the loop of Henle This theory has since been rejected by several authors (Stephenson 1965 Marsh et al 1967)

[1] In a recent study by Ja[illegible]n et al 196[illegible] it was found that the osmolarity of descending vasa recta blood at normal flow w[illegible] hypo[illegible] [illegible]lati[illegible] its environment while at low flow rates the blood attained equil[illegible]

C Intrarenal blood flow distribution

The first quantitative data on the blood flow distribution to the different regions of the kidney were provided by Kramer and coworkers (1960) using a photoelectric technique From the data obtained by this method Ullrich et al (1961 a) summarized that about 90 % of the total amount of blood perfusing the kidney was distributed to the cortical glomeruli and the peritubular capillary systems and thus about 10 % to the juxtamedullary glomeruli and the vasa recta Only about 1 % was distributed to the inner medulla

Values of the same order of magnitude were obtained by Lilienfield and co workers (see Lilienfield and Pomerantz 1963) using the accumulation rate of I^{131} labelled albumin as an index of medullary blood flow

Hársing and Pelley (1965) using the Rb^{86} accumulation method obtained values which on the whole are in agreement with those of Kramer et al and Lilienfield et al in spite of the fact that this method is to be regarded as a method for the determination of effective regional blood flow

The effective regional blood flow as determined by inert gas methods (for summary see Aukland 1964) is as regards the cortex, roughly the same as the absolute flow The medullary and especially the inner medullary blood flow is generally smaller than the absolute flow

As concerns the regional blood flow as related to *perfusion pressure* and to *type* and *degree of diuresis* which are the topics of the present investigation very little is known Thurau Deetjen and Kramer (1960) using a photoelectric technique found that the cortical blood flow remained unchanged when the perfusion pressure was varied from about 80 mm Hg to about 200 mm Hg that is to say the cortical blood flow was found to be autoregulated The medullary blood flow however was found to be linearly related to the perfusion pressure and thus not autoregulated It should be pointed out that the medullary blood flow in their investigation refers primarily to inner medullary blood flow The increase in urine production and decrease in urine osmolarity found on elevation of perfusion pressure was considered to be attributable to the increased medullary blood flow (Thurau and Deetjen 1962)

Aukland (1966b) found that the wash out rate of hydrogen from the outer medulla remained unchanged at perfusion pressures varying between 100 mm Hg and 160 mm Hg thus indicating an autoregulation of medullary blood flow as was also shown for the total renal blood flow

Gilmore (1964) calculated the regional renal blood flow from the build up time of cold in different regions of the kidney after a slug injection of cold saline into the renal artery and reported that his results confirmed those of Thurau Deetjen and Kramer No results were presented however and this study is therefore beyond the scope of criticism

With regard to osmotic diuresis Thurau Deetjen and Kramer (1960) found the medullary blood flow to be increased in glucose induced osmotic diuresis whilst the cortical blood flow remained essentially unchanged Goldberg and Lilienfield

The vasa recta, which also form a counter current system, are generally held to act as a passive counter current exchange system. Such systems have been described previously for the maintenance of high oxygen tensions in the swim bladder of deep sea fish (Scholander 1954) and for the conservation of heat in the sloth (Scholander and Krog 1957). The functional significance of the vasa recta counter current exchange system in the kidney is that it creates a barrier to the net transport of diffusible substances along the long axis of the vasa recta limbs. Solute removed from the hyperosmotic medullary interstitium by ascending vasa recta blood, which thus gains a high solute concentration, will be passively transported to the descending vasa recta which have a low solute concentration. The amount of solute shunted in this way is dependent upon the existing concentration difference, the spatial arrangement of the vasa recta and the diffusion characteristics of the solute in question. The amount carried away by the blood is proportional to the concentration difference and the blood flow. Water as well as substances (e.g. oxygen) carried by the arterial blood to the medullary regions will be shunted in the opposite direction. This problem has been treated theoretically by several investigators (Scholander 1954, Berliner et al. 1958, Ullrich et al. 1961a, Gunzler in Thurau and Deetjen 1962). A more general theoretical treatment was given by Niesel and Roskenbleck (1963), who pointed out that the efficiency of a counter current exchange system is greatly reduced when the outflow exceeds the inflow in the system.

There is in fact experimental evidence of a highly effective counter current exchange system in the kidney. Micropuncture studies of Wirz (1953), Gottschalk and Mylle (1959) and Ullrich et al. (1961 b) have shown that the osmolarity of vasa recta blood is essentially the same[1] as that of the fluid in the tubuli and collecting ducts at the same level in the medulla. The slow uptake of diffusible substances such as sodium, water and krypton (Morel et al. 1960, White et al. 1961, Lassen and Longley 1961) as well as the low inner medullary and urinary oxygen tension (e.g. Rennie et al. 1958, Aukland and Krog 1961, Aukland 1962, Ulfendahl 1962 a, Aperia and Liebow 1964) also support this finding.

An alternative hypothesis that the vasa recta system acts as a counter current multiplier, the driving force being the hydrostatic pressure difference between the vasa recta limbs, has been outlined by Lever (1965). This hypothesis has been criticized recently by Berliner and Bennet (1967), who emphasize the improbability that the hydrostatic pressure is able to create the high solute concentrations found in the medulla.

Pinter and Shohet (Pinter and Shohet 1963, Shohet and Pinter 1964) have outlined a theory in which the high solute concentrations in the inner zone could be created by the vasa recta system (or by the loops of Henle) when the counter current multiplier is limited to the thick ascending part of the loop of Henle. This theory has since been rejected by several authors (Stephenson 1965, Marsh et al. 1967).

[1] In a recent study by Jan[illegible]on et al. (1967) it was found that the osmolarity of descending vasa recta blood at normal flow was hypotonic in relation to its environment, while at low flow rates the blood attained equilibrium.

C Intrarenal blood flow distribution

The first quantitative data on the blood flow distribution to the different regions of the kidney were provided by Kramer and coworkers (1960) using a photoelectric technique From the data obtained by this method Ullrich et al (1961 a) summarized that about 90 % of the total amount of blood perfusing the kidney was distributed to the cortical glomeruli and the peritubular capillary systems and thus about 10 % to the juxtamedullary glomeruli and the vasa recta Only about 1 % was distributed to the inner medulla

Values of the same order of magnitude were obtained by Lilienfield and co workers (see Lilienfield and Pomerantz 1963) using the accumulation rate of I^{131} labelled albumin as an index of medullary blood flow

Harsing and Pelley (1965), using the Rb^{86} accumulation method, obtained values which on the whole are in agreement with those of Kramer et al and Lilienfield et al in spite of the fact that this method is to be regarded as a method for the determination of effective regional blood flow

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(1963) found however that the accumulation of I^{131} labelled albumin in the medulla remained essentially unchanged in mannitol induced osmotic diuresis

Concerning the pertinent question of the action of ADH (antidiuretic hormone) Thurau Deetjen and Kramer (1960) found that the medullary blood flow was increased in water diuresis in relation to antidiuresis Infusion of ADH in concentrations elevating the systemic blood pressure less than 10 mm Hg caused a marked fall in medullary blood flow parallel to an increase in urine osmolarity In a review by Thurau (1964a) it was reported that in numerous unpublished experiments by Kramer and his group no vasoactive effect of ADH at physiological concentrations on medullary vessels could be demonstrated This latter finding was supported by Aukland (1966b) who found no noteworthy change in the wash out rate of hydrogen from the outer medulla after the infusion of ADH in concentrations elevating the systemic blood pressure less than 10 mm Hg

Grangsjo and Persson (1968) in an extensive study on the influence of vasoactive substances on the regional renal blood flow using a heated thermocouple technique found that at physiological concentrations ADH caused only small and inconsistent changes in the medullary blood flow

Harsing and Pelley (1965) using the Rb^{86} accumulation technique found the medullary blood flow to be decreased in severe dehydration but this is not necessarily due to the action of ADH

Fourman and Kennedy (1966) employing a method in which the degree of staining of the vessel wall after an injection of a fluorescent dye was used as an index of blood flow found that ADH and severe dehydration reduced the medullary blood flow

In addition to the studies reviewed above numerous investigations on the regional renal blood flow have been carried out with the use of the PAH extraction method and methods in which the back diffusion of urea, negative free water clearance and urine oxygen tension (see Aukland 1962 1964) have been taken as indices of the medullary blood flow Since on *a priori* grounds these methods cannot be accepted as methods for regional renal blood flow determination and since there is no acceptable independent verification of their validity the results obtained by the methods cannot be accepted with confidence

CHAPTER 3

Indicator and detecting system

In this work a new indicator dilution technique is presented for analysis of the regional circulatory conditions in the kidney P^{32} labelled red cells are used as the indicator the activity being monitored either with beta sensitive needle shaped semiconductor detectors inserted in the renal parenchyma or end window semi conductor detectors placed on the surface of the kidney The pulses from the detectors are amplified with charge sensitive preamplifiers and linear amplifiers and are recorded on scalers with automatic print out

A Indicator

P^{32} labelled red cells have been used extensively in circulation research since P^{32} as a cell label was introduced by Hahn and Hevesy (1940) One disadvantage of P^{32} phosphate however is that it leaks from the red cells Lawson (1962) found the average loss was 5 25 % per hour in 41 intact dogs anaesthetised with barbiturates, and 5 88 % per hour in 31 splenectomized dogs anaesthetised with barbiturates Reeve et al (1953) found even higher values

In this investigation P^{32} labelled red cells were used as the indicator because P^{32} is a pure beta emittor with an energy (1 7 MeV)[1] suitable for the present purpose

Labelling procedure

A citrate phosphate buffer solution according to Mollison et al (1958) was used as the labelling solution This consisted of

Trisodium citrate	3 0 g
Sodium di hydrogen phosphate ($NaH_2PO_4 2H_2O$)	0 015 g
Glucose	1 0 g
Water to 100 ml	

The idea of using a citrate solution was that this ion would enhance the uptake of P^{32} phosphate

2 ml red cells were suspended in 0 2—0 5 ml of the citrate phosphate solution to which about 15—20 mC (millicurie) of carrier free P^{32} phosphate[2] had been added The suspension was incubated at 38 C for about 2 hours after which time 60 % or more of the label was found to have become incorporated in the cells The cells were then washed 3—4 times with isotonic NaCl solution at room temperature and

[1] Information on the type and energy of the radioactive radiation from isotopes mentioned in this work has been taken from the Handbook of Chemistry and Physics 44th edition 1962—63 Chemical Rubber Publishing Co Cleveland Ohio USA

[2] The P^{32} phosphate solution was obtained from AB Atomenergi Studsvik Sweden

resuspended in 2 ml of their own plasma The activity in plasma was then found to be less than 1 % of the activity in the red cells

The red cells were then washed once again during the experiment if this lasted for more than 1 hour In measurements of internal recirculation for example or other measurements in which the extracellular activity had to be as low as possible, the red cells were washed immediately before the injection

B Detecting system

a Detectors

The detectors used in this study were semiconductor detectors The principle of action of these detectors is similar on the whole to that for ionization chambers but with the important difference that the medium between the cathode and anode consists of a semiconducting material silicon or germanium instead of a gas The detecting process involves absorption of the radioactive radiation in the medium with production of high energy secondary electrons which in turn give further ionization with the formation of free electrons and positive so called holes The process continues until no electron has sufficient energy to cause further ionization In the presence of an electric field across the medium these charge carriers give rise to a current pulse The charge of this pulse is proportional to the energy which has been absorbed in the medium Creation of an ion pair in silicon requires about 3 5 electron volts For comparison it may be mentioned that the value for gases is of the order of 30 electron volts per ion pair

The collection time in the semiconductor detector is very short due to the high and approximately equal mobility for free electrons and holes The rise time of the current pulse can thus be reduced to an order of nanoseconds

One problem concerning the so called homogeneous detector described above which is in principle the simplest type of semiconductor detector is the difficulty in producing crystals with a sufficiently high degree of purity so that the life span for the free charges is long in relation to the collection time and also that the leak current is small These problems have been partly overcome by the construction of detectors with *n p* junctions or, in order to obtain larger sensitive volumes, with *n i p* junctions

Some important properties of semiconductor detectors are

1 high resolving ability compared with NaI crystals
2 high sensitivity per detecting volume unit compared with gas filled detectors
3 that it is possible to make them in different sizes and shapes and
4 long life span even with small dimensions The last point is important in the present connection since the main disadvantage of small Geiger Muller tubes is their short life span For further details reference may be made to Dearnaley and Northrop (1966

In the present study several different types of semiconductor detectors were used (Figs 2 a and b) Fig 2 a shows three types of end window detectors without capsules

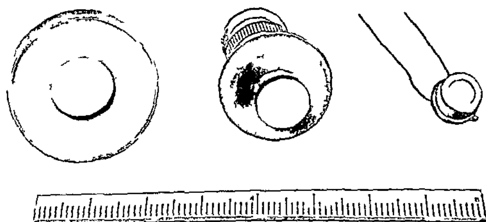


Fig 2a Three different types of end window semiconductor detectors used in the investigation Seen from the left they are a surface barrier semiconductor detector a lithium drifted *n i p* detector and a phosphor diffused type

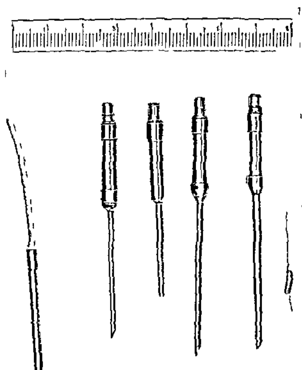


Fig 2 b The figure shows some of the needle detectors used On the left is shown a surface barrier type enclosed in a stainless steel tube The detectors to the right are all lithium-drifted *n i p* detectors enclosed in stainless steel or aluminium tubes with outer diameters varying between 1 0 and 2 5 mm A bare 2 mm crystal (the actual detector) is seen to the extreme right

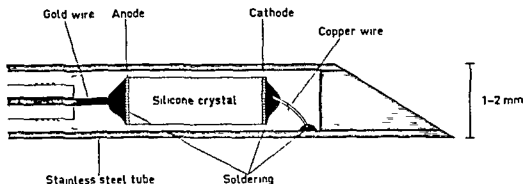


Fig. 2 c. Cross section of a lithium drifted *n i p* needle detector

Seen from the left they are a surface barrier semiconductor detector (AB Atomenergi, Studsvik, Sweden), a lithium drifted *n i p* detector (AB Atomenergi, Studsvik, Sweden) and a phosphor diffused type (SJ2500, RCA Victor, Ltd, Canada). All three types have been found practicable for *in vivo* detection. The phosphor diffused detector was used most. During use the detectors are shielded electronically and mechanically by an aluminium capsule with an aluminium window 15 μ thick.

Fig. 2 b shows some of the needle detectors which have been used. On the left of the figure is a surface barrier type enclosed in a stainless steel tube 2 mm in diameter and with a wall thickness of about 60 μ. The actual crystal is 1 mm wide and 10 mm long and is oriented in one direction. This detector has been developed in collaboration with Mr Madagbas, Department of Physics, Uppsala, Sweden. The needle detectors[1] which were generally used are shown on the right of the figure and consist of 2.5—4.0 mm long lithium drifted *n i p* silicon crystals enclosed in stainless steel or aluminium tubes with a diameter of 1.0—2.5 mm. A cross section of one of these detectors can be seen in Fig. 2 c. These will be referred to hereafter as ND 1 0, ND 1 5 and so on. The thickness of the sheath wall in the sensitive part of the needle detector or, as in the third and fourth detectors in Fig. 2 b, along the entire needle, is of the order of 50 μ. The latter tubes were obtained from Uniform tubes Inc., Collegeville, Pa 19426, USA. The centres of the crystals in these needle detectors are 6—8 mm from the tip. A bare 2 mm crystal is seen on the right of the figure. The needle detectors described are now available commercially (Ealco AB, Stadsgården 18, Stockholm, Sweden). The detectors were operated with 10—50 Volts.

b. Associated electronics

Two-channel systems which allowed simultaneous monitoring from two detector positions were used. The pulses from the detectors were amplified in charge sensitive

[1] The author is deeply grateful to A. Lauber and B. Rosencrantz, AB Atomenergi, Studsvik, Sweden, for their willing cooperation and skilful work in the development of these detectors.

preamplifiers (Model 100 B Tennelec Oak Ridge, Tennessee USA) and linear amplifiers (RLA 1 Tracerlab Inc, Boston 10, Mass, USA)

The discriminator level was adjusted with a pulse height analyser (Tracerlab RLA 55) so that the background activity was 1—2 cps corresponding to an energy level of approximately 50 KeV (kiloelectron volts)

The pulses were recorded on scalers with automatic print out — in one of the channels on a digital printer (Type DIE Kienzle Apparate GmbH West Germany), allowing up to three readings per second and in the other channel with a decimal pip method (Danielson et al 1963) with recording on a photokymograph (Ultralette, ABEM Solna, Sweden)

For a more detailed description of the linear amplifier and print out components the reader is referred to Sjöstrand (1964)

In some of the later experiments the described linear amplifier and the print out system were exchanged for a two channel system from AB Atomenergi, Studsvik, Sweden, consisting of linear amplifiers (Mod 4615), discriminators (Mod 6212) and ratemeters (Mod 4656) The signals from the ratemeters were fed to the photokymograph

c Properties of the detecting system

Counting rate efficiency

The counting rate efficiency was determined as the counting rate (cpm) from the detectors immersed in a sodium P^{32} phosphate solution with a concentration of 1 μC/ml For the end window detectors the efficiency was approximately 10 000 cpm/μC P^{32}/ml For the needle detectors this value was 5 000 cpm/μC P^{32}/ml for the surface barrier type and varied between 1,500 cpm/μC P^{32}/ml (ND 1 0) to almost 10 000 cpm/μC P^{32}/ml (ND 2 5) for the lithium drifted type

The properties which determine this efficiency for beta emitting isotopes include

1 the size of the sensitive surface of the detector
2 the background noise caused by the leak current through the detector,
3 absorption in the surrounding metal capsule and
4 the energy of the beta radiation

Thus a beta radiation of higher energy gives an approximately proportionally[1] higher counting rate due to the longer range of these beta particles For beta energies lower than P^{32} (1 7 MeV) the counting rate efficiency is thus correspondingly lower For the needle detectors however absorption in the surrounding steel tubes must be taken into consideration A wall thickness of 50 μ gives total absorption of beta particles with lower energy than about 200 KeV (Francis et al 1954) It was found possible however to turn aluminium and titanium tubes on a lathe down

[1] It can be shown that the counting rate efficiency ratio R between two isotopes with maximum energies E_1 and E_2 can be calculated approximately as

$$R \approx \frac{E_1^{\,4}}{E_2^{\,4}}$$

to a wall thickness of 30 μ with acceptable robustness This thickness allows the passage of beta particles with an energy exceeding about 80 KeV (Francis et al 1954)

The electronic noise in the needle detectors at a temperature of 40 C extends to an energy of about 50 KeV which means that radiation which looses less energy than 50 KeV in its passage through the sensitive volume of the detector cannot be detected (Lauber and Rosencrantz 1964)

As a result of the small detecting volume of the detectors the sensitivity to gamma radiation is small (Lauber and Rosencrantz 1964)

Stability

The stability of the detecting system was tested at regular intervals during the course of the experimental series, by determining the coefficient of variation for the recorded pulse rate from a P^{32} source during a period of 2—24 hours When this was determined at the end of the series of experiments the activity was recorded over successive five minute periods during a total time of three and a half hours from two detectors (ND 1 5 and ND 2 0) immersed in a P^{32} solution The coefficient of variation was found to be $\pm 0\ 60$ % for ND 1 5 and $\pm 0\ 90$ % for ND 2 0 The coefficients of variation due to statistical fluctuations of the radioactive decay were estimated at $\pm 0\ 57$ % and $\pm 0\ 34$ % respectively Previous values[1] were of the same order of size In *in vivo* application the mechanical strain is greater than in model experiments but the stability did not appear to change essentially

Linearity

The linearity of the detecting system was determined on several occasions Such a test is illustrated in Fig 3 where the counting rate of two needle detectors immersed in a dilution series of P^{32} in saline solution was determined The values are plotted on a log log scale Perfect linearity starting from the lowest values (concentration = 2) is indicated by the continuous lines At lower counting rates the experimental values fit the line for perfect linearity while at higher counting rates there is a small but distinct discrepancy At the highest counting rate i e about 200 000 cpm, the discrepancy is 5 8 % for ND 2 0 and 10 4 % for ND 1 5 The cause of this counting loss lies presumably in coincidence loss in the associated electronics The detector itself can be excluded however as a causative factor (see p 16) For the experiments of the present study this count loss is negligible

Monitored volume

To obtain an idea of the size and form of the monitored volume measurements of the steric efficiency pattern of the needle detectors with P^{32} as the radioactive source were carried out

The experiments were conducted by moving (with a micromanipulator) a small

[1] The detectors have been successively developed during the course of the experimental series Some of the earlier detectors used had a low efficiency and were not stable

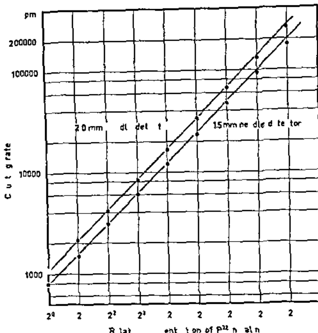


Fig 3 Analysis of a d lution series of P phosphate in 0 9 saline solution with two needle detectors (ND ? 0 and 1 5) The data are represented in log log scale A perfect linearity starting from the lowest values (concentration = 2) is indicated by the straight lines At lower counting rates the experimental values fit the lines for perfect linearity while at higher counting rates there is a small but distinct discrepancy of 5 8 and 10 4 for ND 2 0 and ND 1 5 respectively at about 200 000 cpm

($\sim$1 mm^2) P^{32} source into different positions in the vicinity of the needle detector and measuring the counting rates The source and detector were immersed in isotonic NaCl solution The measuring programme was as follows The radioactive source was first placed close to the surface at the tip of the needle detector and the counting rate determined The source was then moved away in half millimeter steps in a direction normal to the surface of the detector up to a final distance of 7 mm at which distance no counts were recorded The procedure was then repeated along parallel lines at 1 mm intervals i e at 1 mm 2 mm 3 mm etc from the tip of the detector From the data thus obtained the mean counting rate was first calculated between 0 and 0 5 mm 0 5 and 1 0 mm 1 0 and 1 5 mm etc from the surface of the detector at 1 mm 2 mm etc from the tip of the detector These values were calculated by simple interpolation This mean counting rate was considered to be the mean counting rate within a small volume segment with an arbitrary area of 1 × 1 mm and with a thickness of 0 5 mm Fig 4 shows four such volume segments located at a mean distance r from the central axis of the detector and at a certain distance from the tip If the detector had been immersed in a P^{32} solution with the concentration so adjusted that the amount of radioactivity in such a volume segment was the same as that in the source then the counting rate from each segment would be the same as the mean counting rate from the source But there are $2\pi r$ (see Fig 4) such segments the total volume of which is the volume of a rotation body at the distance r from the central axis of the detector and cross section dimensions 1 0 × 0 5 mm (see Figs 4 and 5 below the main diagram) The counting rate arising from the radioactivity within this rotation body is thus obtained

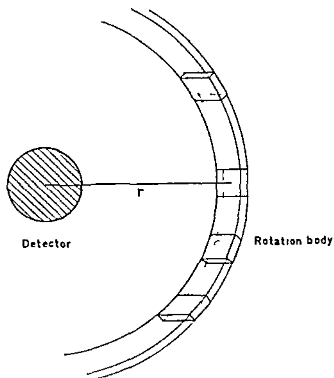


Fig 4 The figure shows a part of a rotation body located at a certain distance from the tip of the detector and at distance r from the central axis of the detector The cross section area is 0 5 × 1 0 mm The rotation body can be regarded as being made up of 2πr small volume segments having the dimensions 1 0 × 1 0 × 0 5 mm 4 such segments are shown in the figure When the detector is submerged in a P^{32} solution the number of counts arising from the rotation body is 2πr times the number of counts per time unit arising from one such volume segment which in turn is calculated by interpolation between the values obtained from a P^{32} source moved into different positions in the vicinity of the detector See also Fig 5

by multiplying the mean counting rate by 2πr The total counting rate recorded by the detector is obtained by adding the counting rates from all such rotation bodies The counting rate from any rotation body can now be expressed in per cent of the total counting rate This is the number given in the rotation body in Fig 5 below the main diagram The values above the detector in Fig 5 are the percentage of the total counting rate arising from all the different rotation bodies Dashes (—) represent values less than 0 05 % Corresponding iso counting rate lines are shown on the lower side of the detector The column on the left gives the percentage number of counts obtained from 0 5 mm thick layers around the detector parallel with its longitudinal axis In the same way the boxed row numerals in the upper part of the figure give the number of counts obtained from 1 mm thick planes cut perpendicularly to the longitudinal axis of the detector

The detector efficiency as visualized in the figure will be referred to in the following as the steric efficiency pattern of ND 1 5 for P^{32}

The maximal range in water for a 1 7 MeV beta particle is about 8 mm This value is an upper limit of the radius of the detecting volume (correction for absorption in the surrounding steel capsule etc will reduce it somewhat) As evident from the figure in practice the monitored volume is smaller and at the same time less well defined The monitored volume may instead be defined as that volume included within a certain boundary surface *while at the same time* the number of counts arising from the volume expressed as percentage of the total counts (arising from an

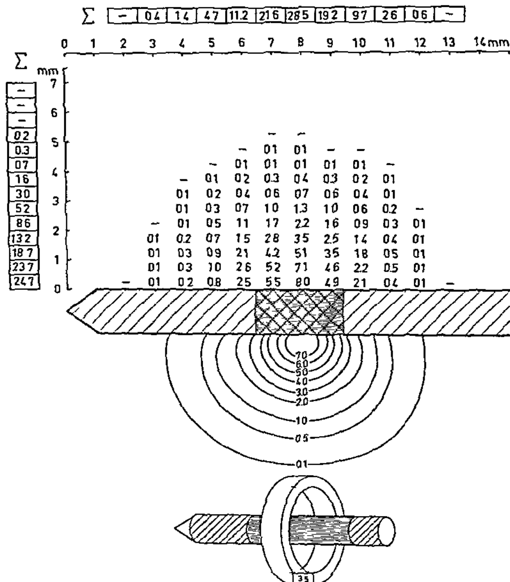


Fig 5 The figure illustrates the steric efficiency pattern of ND 1 5 for P³² The *numerals above* the detector denote the number of counts per time unit arising from the different rotation bodies around the detector in per cent of the number of counts arising from all such rotation bodies Such a rotation body is illustrated below the main diagram The lines drawn below the detector connect rotation bodies giving rise to the same number of counts per time unit ("iso-counting rate lines)

The column to the left gives the percentage number of counts arising from 0 5 mm thick layers around the detector parallel with its longitudinal axis The boxed row of numerals in the upper part of the figure denotes the counts arising from *1 mm thick planes* cut perpendicular to the longitudinal axis of the detector

infinite volume) is given Thus about 85 90 and almost 100 per cent of the total counts arise from the volumes, which are included within the three outer iso counting rate lines shown in Fig 5

The other needle detectors used in this investigation exhibit, in principle the same type of 'steric efficiency pattern The efficiency pattern of the end window detectors has not been investigated in detail

It was found that almost 25 % of the total number of counts arise from the 0 5 mm thick layer nearest to the surface of the detector i e in that region where a damaged zone due to the trauma of insertion is to be found (see below) With regard to the planes perpendicular to the longitudinal axis of the detector the mean efficiencies are similar in the three 1 mm thick 'planes surrounding the centre of the detector but rapidly decrease to zero with increasing distance from the crystal (see top boxed row of values in Fig 5) More than 90 % of the total number of counts recorded arise from a 5 mm thick plane surrounding the centre of the detector

Both the geometry of the needle detectors and the use of P^{32} phosphate as a red cell label constitute a compromise between several different factors An important consideration in the type of circulation studies reported is that the monitored volume should fit to the geometrical conditions in the organ to be studied — in this case the kidney where the medullary region is of special interest The extent of this volume in the longitudinal axis of the detector is mainly determined by the length of the silicone crystal about 3 mm being chosen here as most suitable (see further p 60) The distance monitored perpendicular to the longitudinal axis of the detector is mainly dependent on the beta energy of the isotope

It is important in this connection that the proportion of activity arising from the layer nearest to the surface of the detector should not be too large as the damaged zone consisting of a non perfused zone i e a zone where the blood flow is zero and also outside this a zone with more or less disturbed blood flow, is to be found in the immediate vicinity of the detector The non perfused zone constitutes in itself no great problem except when the regional blood volume is studied, it does not affect transit time determinations The zone with a disturbed blood flow can possibly give rise to errors in the mean transit times Isotopes with relatively low beta energies e g I^{131} (maximum beta energy 0 60 MeV), and hence small monitored volumes should therefore be used with caution When using diffusible indicators such as Kr^{85} these problems become very complicated In studies of the wash out rate of this isotope values were obtained in the renal cortex, which were considerably lower than expected There may be many reasons for this discrepancy and it is very likely that one of them is to be found in the damaged zone around the detector

With regard to the choice of other indicators the possibility of using some short lived isotopes may be mentioned

C Summary

In the present study on the intrarenal blood flow P^{32} labelled red cells have been used as the indicator, because P^{32} is a pure beta emittor with an energy (1 7 MeV) suitable for the purpose The drawback of P^{32} phosphate as the cell label is that it leaks from the red cells Detection of the beta radiation was performed by end window semiconductor detectors for surface monitoring and needle shaped semi conductor detectors for internal monitoring The pulses from the detectors were amplified by charge sensitive amplifiers and linear preamplifiers and are recorded on scalers with automatic print out

The recording system was found to have good stability and acceptable linearity

To obtain an idea of the size and form of the monitored volume a P^{3} source was moved into different positions in the vicinity of the needle detector It was found that if the detector is immersed in a P^{32} solution almost 25 % of the total number of counts will arise from the 0 5 mm thick layer nearest to the surface of the detector, where the non perfused zone due to the trauma of insertion is to be found The efficiency was roughly the same in the three 1 mm thick planes surrounding the centre of the detector and cut perpendicular to the long axis of the detector More than 90 % of the total number of counts will arise from a 5 mm thick plane surrounding the centre of the detector

It is stressed that the monitored volume should fit to the geometrical conditions in the organ to be studied The extent of this volume in the longitudinal axis of the detector is mainly determined by the length of the silicone crystal (the actual detector) The distance monitored perpendicular to the long axis is dependent on the betaenergy of the isotope in question

CHAPTER 4

On the theory of indicator dilution techniques in the case of internal or external monitoring

Indicator dilution methods for analysis of blood flow and blood volume have been used for more than a hundred years The first experiments were carried out by Hering (1829), who determined the circulation time through the cardiovascular system using potassium ferrocyanide as the indicator The method was further developed by Stewart in a series of studies beginning at the turn of the century (Stewart 1898 1921) making it possible to obtain quantitative values of the cardiac output It was first due to Hamilton and coworkers (Hamilton et al 1928 1932 1947), however, that this technique gained better theoretical and practical anchorage and thereby more extensive use, and today it is a routine method for determination of the blood flow and blood volume both in the cardiovascular system and in individual organs For a more detailed historical review reference may be made to Dow (1956) and Fox (1962)

During recent years this method has been developed further The introduction of radioactive isotopes in biological research permitted a greater selection of indicators for determination of both intravascular and extravascular flows In the so called conventional Stewart Hamilton technique detection of the indicator was performed in blood collected from the efferent vessel The development of equipment for measuring radioactivity has also allowed detection over the organ itself (Conn 1962 Sevelius 1965)

A Present concepts

The present concepts concerning the extensive and much ramified subject of the indicator dilution technique will not be considered in detail here and the following considerations will be limited to the problems surrounding the determination of the so called mean transit time for an intravascularly flowing particle

If the flow Γ and the volume V of intravascular fluid particles in an organ or part of an organ are known the mean transit time $\bar{t}$, can be calculated as follows

$$V = \Gamma \times \bar{t} \qquad (1)$$

This time defined as above will be referred to hereafter as *the true mean transit time*

If the flows of fluid particles through the various routes in an organ are f_1 f_2 f_n and the corresponding transit times are t_1 t_2 t_n the mean transit time can be defined as follows

$$\bar{t} = \frac{f_1 \; t_1 + f_2 \; t_2 + \quad f_n \; t_n}{f_1 + f_2 + \quad f_n} \qquad (2)$$

The mean transit time can be determined experimentally In the conventional Stewart Hamilton technique an indicator representative of the fluid particles whose mean transit time is to be determined is injected as a slug injection into the afferent vessel (continuous injection does not usually give any essential theoretical advantage) From the indicator dilution curve obtained in blood collected from the efferent vessel the true mean transit time can be determined accurately as follows

$$\bar{t} = \frac{\int_0^\infty c(t)\, dt\ t}{\int_0^\infty c(t)\, dt} \qquad (3)$$

where c(t) is the concentration of the indicator in the effluent blood The prerequisites for the validity of this equation are (a) the flow and volume of the fluid particles are constant (b) the distribution of transit times for entering particles of fluid does not change with time (c) the flow of indicator particles is representative of the flow of total fluid that is the distribution of transit times for the indicator particles is the same as that for fluid particles, (d) the system has no stagnant pools (this assumption is needed only for measurement of volume from mean transit time and flow) and (e) there is no recirculation of indicator (Meier and Zierler 1954) This equation was first introduced by Hamilton et al (1932) For further theoretical considerations concerning this field of research reference may be made to Meier and Zierler (1954) Burger et al (1956) van der Feer (1958) Zierler (1958) Zierler (1962) and Grodins (1962)

The determination of the mean transit time by use of detectors *over or within the organ* is more complicated and it has been found impossible hitherto in general to determine the true mean transit time Under certain circumstances however it can be shown that the time $\bar{t}_{A/H}$ obtained by dividing the area of the indicator dilution curve by its greatest height corresponds to the true mean transit time This relationship has been inferred on the basis of different theoretical approaches by several authors (Zierler 1965 Powers and Sevelius 1965 Høedt Rasmussen et al 1966) Kramer and coworkers in their studies of the regional renal circulation have determined the mean transit time using equation (3), i e the indicator dilution curve has been analysed as if it were a conventional Stewart Hamilton function This method would seem to be incorrect from theoretical considerations (see below)

In the present studies of the renal circulation the methods described above were not adequate The prerequisites for determination of mean transit time as $t_{A/H}$ cannot *a priori*, be considered to be fulfilled Kramer s method, on the other hand lacks an acceptable theoretical foundation It was therefore necessary to reappraise the theoretical basis of the indicator dilution technique in organ monitoring with a view to (a) investigating the validity of the methods discussed above, and (b) seeking other more suitable methods if necessary

B Generation of the indicator dilution curve in the case of internal or external monitoring

A detector for radioactivity placed in, or over an organ will monitor the radioactive decay in a more or less well defined volume around the detector In beta detection

this volume is relatively clearly defined and is *indicated by the maximal beta energy* of the isotope in question We now assume that within the whole monitored volume there are *n* pathways for the indicator particles (and the fluid particles) A pathway can consist of a capillary, or part of a capillary or a large vessel *A pathway is defined as a route through which the transit time for the indicator and the fluid particles has one unique value*

The volume in one of these pathways is v_i, where the index i represents the numerals 1 2 n 1, n Similarly the flow through it is f_i and the transit time t_i The total volume of all pathways is V, the flow F and the mean transit time t_i The efficiency (counting rate per unit activity, e g cpm per μC) with which the detecting system records a radiation source within volume v_i is dependent on the position in relation to the detector the absorption conditions in the medium and the properties of the detector itself The latter factors are regarded as constant The efficiency e_i, is then expressed as a function of the position of the radiation source in the space

$$e_i = f(x, y, z) \tag{4}$$

This position is in turn a function of the time after the indicator has been injected The efficiency can thus be expressed as a function $g_i(t)$ of time t

$$e_i = g_i(t) \tag{5}$$

In the artery of the organ a certain amount of indicator M_{tot} is injected as a slug injection The flow in this artery of the fluid particles, which the indicator represents is denoted as F_{tot} The amount of indicator M which passes to the monitored region in a single circulation is governed by the equation

$$M = \frac{M_{tot} \; F}{F_{tot}} \tag{6}$$

and in the same way the amount of indicator m_i which passes to a single pathway is calculated (the equation assumes that the behaviour of the slug faithfully reflects that of an equivalent amount of blood in the arterial inflow)

The recorded counting rate $i_i(t)$ from the indicator in the pathway v_i is then obtained as

$$i_i(t) = m_i \; e_i = \frac{M_{tot}}{F_{tot}} \; f_i \; g_i(t) \tag{7}$$

The recorded number of counts $\int_0^\infty i_i(t)dt$ which have arisen from the indicator during its transit through the monitored volume in the pathway v_i is obtained by integration with respect to time

$$\int_0^\infty i_i(t)dt = \frac{M_{tot}}{F_{tot}} \; f_i \int_0^\infty g_i(t)dt = \frac{M_{tot}}{F_{tot}} \; f_i \; t_i \frac{\int_0^\infty g_i(t)dt}{t_i} \tag{8}$$

The expression $f_i \; t_i = v_i$ (eq 1) and $\frac{\int_0^\infty g_i(t)dt}{t_i}$

equals the mean efficiency, e with which the detector will record the indicator during its transit through the volume v_i or in other words the efficiency for localization of the indicator at every position within the volume v_i. This means that if a unit amount of activity has been distributed into the volume v_i then the counting rate would be just e (Note that efficiency has been defined here as the counting rate (cpm) obtained from a unit amount of activity (μC)) Equation (8) can now be written as

$$\int_0^\infty g_i(t)dt = \frac{M_{tot}}{F_{tot}} \; v_i \; e_i \tag{9}$$

The formula can also be derived by means of integration with respect to volume. The total number of recorded counts in a single circulation A (area of the recorded curve) is then obtained as

$$A = \frac{M_{tot}}{F_{tot}} \sum_{i=1}^{i=n} v_i \; e_i = \frac{M_{tot}}{F_{tot}} \; V \; \overline{E} \tag{10}$$

where $\overline{E}$ is the mean efficiency with which the detector will record the indicator evenly distributed into the intravascular volume V within the monitored volume, V_{mon}.

This efficiency equals the efficiency $\overline{E}_0$ operating when the indicator is evenly distributed in the monitored volume providing that the intravascular volume is evenly distributed in the monitored volume. This prerequisite is not completely fulfilled in the present investigations since the region nearest to the detector consists of a non perfused zone in which the indicator cannot be distributed.

The factors $\overline{E}$ and V in equation (10) cannot be determined directly. For the further evaluation equation (10) is therefore rearranged to yield

$$A = \frac{M_{tot}}{F_{tot}} \; \frac{C \; V \; \overline{E}}{C \; V_{mon} \; E_0} \; E_0 \; V_{mon} \tag{11}$$

where C is the concentration of the indicator after the indicator has been equilibrated throughout the total blood volume.

Here $C \; V \; \overline{E}$ equals the counting rate recorded from the detector in the parenchyma after the indicator has been equilibrated throughout the total blood volume and $C \; V_{mon} \; \overline{E}_0$ the counting rate obtained from the same detector immersed in blood drawn from the animal on the same occasion (Note that different efficiency factors are operating in the two determinations of the counting rate) The ratio between these two counting rates is the experimentally determined regional blood volume[1], $V_{e\,p}$ expressed as a fraction of tissue volume. If $\overline{E}$ equals $\overline{E}_0$ i e when there is no

[1] The indicator is considered the whole time to be representative of blood particles

non perfused zone the ratio between the counting rates equals the true regional blood volume expressed as a fraction of tissue volume, $\frac{V}{V_{mon}}$

In chapter 3 the efficiency $\overline{\Gamma}_{exp}$ was determined as the counting rate recorded from a detector immersed in a solution having a concentration of 1 μC/ml of the beta emitting isotope in question In the monitored volume there will be 1 V_{mon} μC of the isotope $\overline{L}_{exp}$ is then obtained as

$$\overline{L}_{exp} = 1 \; V_{mon} \; \overline{L}_o \tag{12}$$

Equation (11) can now be written as

$$A = \frac{M_{tot}}{\Gamma_{tot}} \; V_{exp} \; \overline{E}_{exp} \tag{13}$$

In the experiments in the present study P^{32} labelled red cells were used as the indicator This indicator is strictly speaking only representative of red cells This does not however alter the above equation since both V_{exp} and Γ_{tot} have to be multiplied by the arterial haematocrit in order to yield regional red cell volume and total red cell flow

If now M_{tot} is known Γ_{tot} i e the flow where the indicator is injected, can be determined In intravenous injections the indicator is mixed in the heart before reaching the kidney Γ_{tot} will in this case therefore denote the cardiac output

In the present investigation this method of determination has been used for the determination of the total flow in the renal artery and in some cases the cardiac output It has been found to be of limited value for absolute determinations, however since the prerequisite of *ideal behaviour of the indicator* has probably not been completely fulfilled (The prerequisite of so called complete mixing)

As evident from equation (10) the greatest part of the curve arises from those pathways which have a *large blood volume* and in which the *efficiency factor is large* If thus the monitored region includes a vessel with high flow (and high activity see eq 6) those counts which arise from the indicator here will not be proportional to the flow but rather to the blood volume of the vessel and the efficiency with which the indicator within the vessel is recorded

C Determination of mean transit time

In this section three different methods for determination of the true mean transit time will be discussed The discussion will be based both on theoretical considera tions and on studies of a hypothetical model In method I the mean transit time is determined as the time $t_{A/H}$ obtained by dividing the area of the indicator dilution curve obtained during a single circulation by its greatest height Method II involves determination of the mean transit time as the time co ordinate for the centre of gravity of the curve t_{cg} i e the curve is trea ed as a conventional Stewart—Hamil

ton function (see eq 3) In method III the mean transit time is determined as that time t_m when half the area of the indicator dilution curve has been recorded $\bar{t}_{A/H}$ supposedly represents the true mean transit time through the monitored region itself while t_{cg} and t_m on the other hand are supposed to represent the mean transit time from the site of injection and through half the intravascular volume within the monitored volume

a $\bar{t}_{A/H}$

At the time point when the indicator dilution curve reaches its peak value $I(t)_{max}$ or H_{max} a certain not necessarily maximal, fraction K_M M of the indicator is present within the monitored volume The efficiency with which the detecting system records this activity can be written as K_E $\bar{E}$ Now it follows that

$$H_{max}=I(t)_{max}=M\ K_M\ \bar{E}\ K_E=\frac{M_{tot}}{F_{tot}}\ F\ K_M\ \bar{E}\ K_E \qquad (14)$$

Combining equations (10) and (14) and solving for V/F give

$$\frac{V}{F}=\frac{A\ K_M\ K_E}{H_{max}} \qquad (15)$$

The expression V/F gives the true mean transit time $\bar{t}$ *through the monitored region* (see eq 1) The relationship between the true mean transit time and the mean transit time $\bar{t}_{A/H}$ which is calculated by dividing the area A of the indicator dilution curve by its height H_{max}, is then obtained as

$$t_{A/H}=\frac{t_1}{K_M\ K_E} \qquad (16)$$

The factors K_M and K_E obviously cannot in general be determined directly and vary with the circulatory conditions, monitored volume steric efficiency pattern etc The prerequisites for factors K_M and K_E to be unity i e for $t_{A/H}$ to give a correct value for the true mean transit time are that all indicator is present within the monitored volume at the time when the peak is recorded and that the indicator is then evenly distributed within this volume or alternatively that the actual efficiency at this time is equal to the mean efficiency If the indicator is injected close to the monitored volume it does not seem improbable that the first criterion is fulfilled On the other hand that at this time the indicator should have an even distribution within the monitored volume or alternatively a distribution such that the efficiency is equal to the mean efficiency $\bar{E}$ is a condition that on *a priori* grounds is unlikely to be fulfilled with the needle detectors used

It may be of interest here to consider in what circulatory system $\bar{t}_{A/H}$ would probably give an accurate value of the true mean transit time In such a system the transit times in afferent vessels to the different capillary regions would be equal, or short

compared to the transit times in the capillaries Further the efficiency factor must, on the average, be smaller for the indicator during the transit through the afferent vessels than when the indicator is in the capillary system Such a system does not seem unrealistic (e g brain and external gamma detection)

b $\bar{t}_g$

With a uniform distribution of the intravascular volume it can easily be shown that the centre of gravity for each part of the indicator dilution curve that arises from an individual pathway will on the average, correspond to that time when the indicator has passed *through half the pathway* volume if (1) the pathway passes straight through the monitored volume or (2) the capillary volume is large in correspondence to the volume in afferent and efferent vessels within the monitored volume, or (3) if the volume in afferent vessels is equal to that in the corresponding efferent vessels [1]

This does not mean necessarily that the time co ordinate for the centre of gravity of the whole indicator dilution curve t_{cg} corresponds to the time when the indicator has passed through half the blood volume in the entire monitored volume Using equations (3) (9) and (10) this can be shown as follows

$$t_{cg} = \frac{\frac{M_{tot}}{F_{tot}} \sum_{1}^{n} v_i \; e_i \; \tau_i}{\frac{M_{tot}}{F_{tot}} \; V \; \bar{E}} \qquad (17)$$

where τ_i is the transit time for the indicator from the injection site to the monitored region and through half v_i Using the relationships $v_i = f_i \; t_i$ and $V = F \; \bar{t}_i$ we obtain

$$t_{cg} = \sum_{1}^{n} \frac{f_i \; \tau_i}{F} \frac{e_i}{\bar{E}} \frac{t_i}{\bar{t}_i} \qquad (18)$$

If all $e_i = \bar{E}$ and all the individual transit times in the monitored volume are equal, equation (18) is reduced to

$$t_{cg} = \frac{\sum_{1} f_i \; \tau_i}{F} \qquad (19)$$

It is thus obvious that $\bar{t}_{cg}$ under these conditions *really* equals the true transit time (see eq 2)

[1] If for example the efferent vess ls have a larger volume than the afferent vess ls the centre of gravity of the curve arising from a long pathway loop extending beyond the centre of the detector will represent an overestimate of the trans t time through half the pathway volume and the centre of gravity of a curve arising from a short loop an underestimation Consequently $\bar{t}_g$ will over estimate the true mean trans t time This condition will not be considered in detail here

We may now conclude that t_{cg} is not in general identical with the true mean transit time. Under most conditions $\bar{t}_{cg}$ will overestimate the true mean transit time since the ratio $\frac{t_i}{\bar{t}_i}$ on the average, exceeds unity.

This conclusion means that

$$\frac{\sum_1^n t_i}{n} > \frac{\sum_1^n f_i t_i}{\sum_1^n f_i} \tag{20}$$

$$f_1\left(\sum_1^n t_i - n\ t_1\right) + f_2\left(\sum_1^n t_i - n\ t_2\right) + f_3\left(\sum_1^n t_i - n\ t_3\right) \\ + f_{n-1}\left(\sum_1 t_i - n\ t_{n-1}\right) + f_n\left(\sum_1^n t_i - n\ t_n\right) > 0 \tag{21}$$

In a long pathway loop the volume is usually large and the resistance high, which means that the flow in the pathway is low and that the transit time is long. For the further discussion it is, however, only necessary to assume that the volumes in the different pathways are equal to state that if

$f_1 > f_2 > \quad > f_n$ then

$t_1 < t_2 < \quad < t_n$

In equation (21) the sum of all terms within the parentheses is 0. The first parentheses are positive, somewhere in the middle of these series the parentheses are near to zero and at the end of the series they are negative. Since $f_1 > f_2 > \quad > f_n$ the whole series have a positive value thus exceeding zero.

c $\bar{t}_m$

At time t_m the indicator in a certain pathway has passed through half its volume, in other pathways less and in others more. The volume which has been passed at time t_m can then be expressed as $^1/_2\ v_i + \Delta v_i$ where Δv_i can be positive, zero or negative. *The mean efficiency up to time t_m is* written analogously as $e_i + \Delta e_i$. Utilizing equation (10) and the definition of $\bar{t}_m$ (that half the number of counts has been recorded at this time) we obtain

$$\frac{A}{2} = \frac{M_{tot}\ V\ \bar{E}}{F_{tot}\ 2} = \frac{M_{tot}}{F_{tot}} \sum_1 [(^1/_2 v_i + \Delta v_i)(e_i + \Delta e_i)] \tag{22}$$

$$\frac{\bar{E}\ V}{2} = \frac{\sum_1 v_i\ e_i}{2} + \sum_1^n (\Delta v_i\ e_i + \Delta v_i\ \Delta e_i + {}^1/_2\ v_i\ \Delta e_i) \tag{23}$$

The first two terms are equal (eq 10) from which it follows that

$$\sum_{1}^{n}(\Delta v_i\ e_i + \Delta v_i\ \Delta e_i + {}^1/{}_2\ v_i\ \Delta e_i) = 0 \qquad (24)$$

It is clearly evident from this relationship that $\sum_1^n \Delta v_i$ is not equal to zero except under certain special conditions for example in those cases where the efficiency is uniform throughout the monitored region

In the following the magnitude of the volume deviation $\sum_1^n \Delta v_i$ will be discussed The percentage error of the mean transit determination can then be calculated by dividing this volume deviation times 100 by half the blood volume (See definition of t_m and eq 1)

Consider first a simple circulatory system where the blood volume is distributed uniformly within the monitored volume The blood volume in the capillary network is considered to be large compared with that in the afferent and efferent vessels within the monitored volume which means that the transit times in the afferent and efferent vessels are short in relation to the capillary transit times and that the number of counts which have arisen from the indicator during its transit in these vessels is negligible The blood flow is expressed as a function $f(x)dx$ of the distance x from an imaginary plane in the periphery of the monitored volume The mean efficiency in a layer situated within the monitored region between x and $x+dx$ from the imaginary plane is $e(x)$ The blood volume within the layer is $a(x)dx$

The indicator is now assumed to reach the monitored volume at a time point t_{app} The number of recorded counts up to time t_m which have arisen from the indicator in the capillaries between x and $x+dx$ within the monitored volume is the product of the amount of the indicator perfusing the capillaries, $M_{tot}\ f(x)\ dx/F_{tot}$ the time $t_m - t_{app}$ and the efficiency $e(x)$ Integration with respect to x gives

$$\frac{M_{tot}\ V\ \bar{E}}{F_{tot}\ 2} = \frac{M_{tot}}{F_{tot}}\ (t_m - t_{app}) \int_x f(x)dx\ e(x) \qquad (25)$$

where the monitored volume extends between x_1 and x_2

The volume deviation, $\Delta a(x)dx$ from the case in which half of the blood volume ${}^1/{}_2\ a(x)dx$ has been traversed by the indicator at time t_m in a layer with a thickness dx located at distance x from the imaginary plane is obtained as

$$\Delta a(x)dx = (t_m - t_{app})\ f(x)dx - {}^1/{}_2\ a(x)dx \qquad (26)$$

Combining these two equations we obtain

$$\Delta a(x)dx = \frac{V\ \bar{E}\ f(x)dx}{2\int f(x)dx\ e(x)} - {}^1/{}_2\, a(x)\, dx \qquad (27)$$

The integrand of all $\Delta a(x)dx$ in the monitored volume between x_1 and x_2 is obtained by integration with respect to x between x_1 and x_2

$$\int \Delta a(x)dx = \frac{V\ \bar{E} \int^{x} f(x)dx}{2 \int^{x} f(x)dx\ \ e(x)} - {}^{1}/_{2} \int_{x}^{x} a(x)dx \qquad (28)$$

But $\int f(x)dx = F$ and $\int a(x)dx = V$ where F and V are as before the blood flow and blood volume in the monitored volume We thus obtain

$$\int \Delta a(x)dx = \frac{V\ \bar{E}\ F}{2 \int_{,} f(x)dx\ \ e(x)} - \frac{V}{2} \qquad (29)$$

The percentage error using t_m for the determination of the mean transit time is then obtained as

$$100 \left[\frac{\bar{E}\ F}{\int f(x)dx\ \ e(x)} - 1 \right]$$

It is evident that the error is dependent on the steric efficiency pattern within the monitored volume and on the blood flow distribution in the region

Fig 6 shows a graphical solution of this error for some hypothetical functions[1] f(x)dx A detector, ND 1 5 is placed 4 mm from an imaginary plane, which in this case is thought to be the cortico medullary border in the kidney The function e(x) was taken from the steric efficiency pattern of ND 1 5 (see p 23) The curves A_1 A_2 B and C give the position of the indicator at time t_m in the capillaries at different levels from the cortico medullary border or in other words the fraction of the capillary volume traversed by the indicator at time t_m

In the linear functions A_1 and A_2 $\int \Delta a(x)dx$ is equal to zero which is obvious from the symmetrical arrangement In function B $\int \Delta a(x)dx$ is negative i e t_m underestimates the true mean transit time The explanation is that if half the area of the indicator dilution curve at time t_m has been recorded in spite of the fact that *less* than half the blood volume has been traversed by the indicator then the average efficiency up to time $\bar{t}_m$ must be greater than the mean efficiency in the entire monitored volume This is actually the case In the capillaries which lie nearest to the centre of the detector where the efficiency is great more than half the blood volume has been traversed by the indicator at time t_m This means that up to time t_m a disproportionately large fraction of the total counts has arisen from the indicator in the capillaries around the centre of the detector i e in a high efficiency region In function C more than half the blood volume has been traversed by the indicator at time t_m i e t_m will give a falsely high value for the mean transit time

The probability that t_m will be a correct estimate of the transit time is however, fairly high If the monitored volume is considered to extend between 1 5 and 6 5 mm t_m in curve B will underestimate the true mean transit time by 2 % and in curve C it will overestimate it by 5 %

In the renal medulla the blood volume in afferent and efferent vessels can hardly be neglected The equations will then be considerably more complicated The furt

[1] An id a of the flow distribution functions used is obtained from the curves in the figure

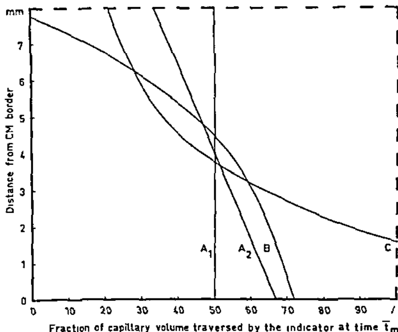


Fig 6 Fraction of capillary volume traversed by the indicator at time t_m in some hypothetical flow distribution functions f(x)dx In the linear curves A and A half the total capillary volume has been traversed by the indicator which means that $\bar{t}_m$ will correctly determine the true mean transit time from the site of injection and through half the blood volume In curve B less than half the total capillary volume has been traversed by the indicator and in curve C more than half of this volume This means that $\bar{t}_m$ will underestimate the true mean transit time in curve B and overestimate it in curve C If the monitored volume is considered to extend between 1 5 and 6 5 mm from the cortico-medullary border the errors in $\bar{t}_m$ are —2 and +5 ° for curves B and C respectively

her evaluation of t_m as a measure of the true mean transit time has therefore been continued on hypothetical model systems

D Studies on hypothetical model systems

The aim of the studies carried out on hypothetical model systems was to investigate the po sibility of determining the true mean transit time in the circulatory system in the kidney especially the medullary region by the methods discussed previously The idea was to investigate the validity of the mean transit time determinations on systems in which the circulatory parameters had been varied within a wide range and to assume that the true conditions in the kidney are to be found somewhere within this range The investigations comprised studies on several series of circulatory systems One of these series will be presented here

In the hypothetical model which is illustrated in Fig 7 the capillary networks in the renal medulla are represented as boxes with afferent and efferent vessels corresponding to descending and ascending vasa recta The blood volume in the

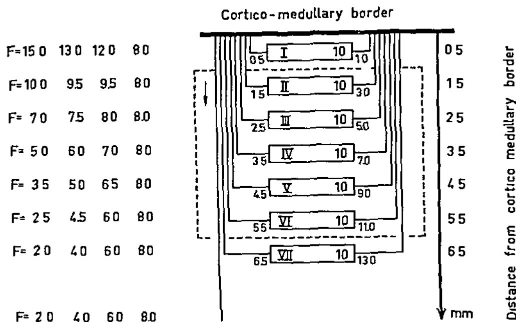


Fig 7 Hypothetical model system used for estimation of the errors in the mean transit time determinations The capillary systems at different levels are represented as boxes with their respective afferent and efferent vessels denoted by straight lines The volume in the capillary system is the same at all levels (10 volume units) The volume in afferent and efferent vessels is proportional to their lengths and twice as great in the efferent as in the afferent vessels (two and one volume units per mm of length respectively) The columns to the left give the 4 alternatives for the flow distribution in the different capillary systems In the first alternative the flow (F) to capillary system I is 15 flow units to system II 10 flow units to system III 7 flow units etc The monitored volume (broken line) is considered to extend between 1 and 6 mm from the cortico medullary border

capillaries is assumed to be equal at all levels starting from the cortico medullary border The blood volumes in the vasa recta are assumed to be proportional to their lengths and twice as great in ascending as in descending vasa recta The distribution of the blood volume between capillaries and vasa recta has been varied according to the following conditions 1 The blood volume is equal in capillaries and vasa recta at a distance of 3 5 mm from the cortico medullary border 2 All the blood volume is in the vasa recta 3 All the blood volume is in the capillaries The blood flow at different levels has been varied from the case in which the flow in the capillary system(I)nearest to the cortico medullary border (see Fig 7) is 7 5 times greater than that in the capillary system lying furthest peripherally (VII) to the situation in which the flow is equal in all the capillary systems The different alternatives for distribution of the flow at different levels are indicated in the columns to the left of the figure In Table I these flow alternatives are expressed as the ratio between the flows in capillaries I and VII

At zero time a P^{32} labelled indicator representative of the blood particles in the

TABLE 1 Errors in the mean transit determination in the hypothetical model systems calculated as t_m, t_{cg} and $t_{A/H}$ in per cent of the true mean transit times

Flow ratio capillary I/V II	$\bar{t}_m$	$\bar{t}_{cg}$	$\bar{t}_{A/H}$	
7.5	+ 4 %	+ 59 %	− 1 %	Blood volume in capillaries and vasa recta equal at a distance of 3.5 mm from cortico medullary border
3.3*	+ 6 %	+ 38 %	− 9 %	
2.0	+ 7	+ 27 %	−26	
1.0	+ 9	+ 20 %	−22 %	
7.5	+63 %	+113 %	+85 %	All blood volume in vasa recta
3.3	+25 %	+ 57 %	+18	
2.0	+12 %	+ 38 %	− 3	
1.0	+ 2	+ 23 %	−14	
7.5	+10	+ 32 %	+ 8 %	All blood volume in the capillaries
3.3	+ 5 %	+ 17 %	− 3	
2.0	− 4	− 1 %	− 1 %	
1.0	− 4 %	− 4	− 4	

system is injected near to the cortico medullary border. The radioactivity distributes in the different descending vasa recta proportional to the flow in these vessels (see eq. 6). The indicator then passes through the different pathways (one pathway consists here of the system descending vas rectum, capillaries and ascending vas rectum) and in each of these it generates an indicator dilution curve, the size and appearance of which depends on the flow and volume and the way in which the volume is distributed within the monitored volume. (The slug in the individual pathway is assumed to be maintained the whole time as an ideal square wave with an infinitely small extension in time.) The efficiency for the indicator in the different vessels has been taken from the steric efficiency pattern of ND 1.5 (see p. 23).

The indicator dilution curve from the whole system is then obtained by adding all these curves. From this total indicator dilution curve the mean transit time is calculated as $t_{A/H}$, t_{cg} and t_m. The calculated t_{cg} and $\bar{t}_m$ values are compared with the true time required for the indicator to pass from the site of injection (at the level of the cortico medullary border) and through half the blood volume in the monitored volume (see Fig. 7). The calculated $\bar{t}_{A/H}$ is compared with the true mean transit time through the monitored volume. Table I shows the results of these comparisons. In the table the error in the calculated mean transit times is expressed as per cent of the true mean transit times. It is evident from the results that the error in the mean transit time calculation with t_m is very moderate for most of the circulatory conditions in the model. As expected, t_{cg} gives falsely high values. $t_{A/H}$ gives moderate errors in both positive and negative directions.

An important point is that in the model system, unlike the kidney, there is no recirculation of indicator from capillary systems located below the monitored volume

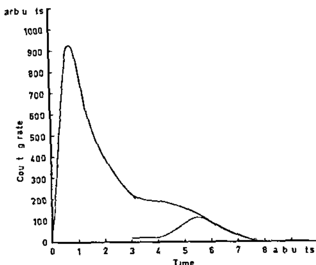


Fig 8 Indicator dilution curve obtained from the model system indicated by an asterisk in Table I The time and counting rate are given in arbitrary units The curve is divided into two parts by semilogarithmic extrapolation of the first part of the curve (broken line) The first part is considered to be generated from the ind cator during its first transit through the monitored volume and the second part from the recirculating indicator from capillary system VII (see Fig 7) The true recirculation curve is indicated by the th nner continuous line The true and calculated recirculation parts agree fairly well with one another both with respect to area and to position in time

with the exception of recirculation from capillary system VII Since however this recirculation occurs at a late stage in the course of the curve it can be identified It is most critical that the recirculation from the regions lying immediately peripheral to the monitored volume should be distinguished Fig 8 shows an indicator dilution curve which has been obtained from the system indicated by an asterisk in Table I This curve was chosen because its appearance is reminiscent of that obtained in the experiments from the corresponding position of the detector in the kidney (see Fig 18a p 57) By semilogarithmic extrapolation[1] of the first part of the curve (broken line) the curve can be divided into two parts The first part is assumed to be generated from the indicator in its first passage through the monitored volume The second part i e that part of the curve which is the difference between the original curve and the extrapolated first part is assumed to be generated by recirculated indicator from capillary system VII The actual curve for recirculation is indicated in the figure by the thinner continuous line The areas of the *calculated* and true recirculation parts of the indicator dilution curve show relatively good mutual agreement as also do the positions in time The recirculation from more peripherally located capillary systems could obviously be identified with even greater certainty

A correct analysis of this curve (in order to determine the flow of blood during its first transit through a region) will include a determination of the mean transit time from the first part of the curve The time determined refers to the transit time for the indicator through the vascular volume with the exception of that volume through which the recirculating indicator passes The intravascular volume determined in one

[1] It has been found empirically both in animal experiments and in most of the model studies that the descend ng part of the first part of the curve falls monoexponentially

way or another must thus be corrected for the latter volume In practice this correction can be made by multiplying the intravascular volume by the factor which is obtained by dividing the area of the first part of the indicator dilution curve by the total area (It has been shown previously that the number of recorded counts is proportional to the intravascular volume in the monitored volume)

In the model series presented here the treatment of the curves described above was not carried out since extrapolation of the curves obtained from the more extreme systems is uncertain Since however the recirculation is so small the deviations from the real values will be negligible Usually a moderately erroneous division of the curve is of relatively minor importance for the final calculations of the blood flow in a region An erroneously high area for the recirculation part for example, will certainly lead to an underestimation of the mean transit time calculated from the first part of the curve but since the corrected volume is also underestimated the final error will be relatively small

E Summary

This chapter deals mainly with the possibility of determining the so called mean transit time in the case of internal detection of an intravascular indicator The generation of the indicator dilution curve is first discussed It can be shown that the area of the curve is proportional to the blood volume in the monitored volume and inversely proportional to the flow rate in the vessel into which the indicator is injected or mixed before it reaches the monitored volume That part of the entire curve area which arises from an individual capillary is, in the same way dependent on both the blood volume in the capillary and the position of the capillary in the monitored volume

For determination of the so called mean transit time three methods have been discussed In the first method the mean transit time is calculated as that time, $t_{A/H}$ which is obtained by dividing the area of the indicator dilution curve by its greatest height In the second method the mean transit time is calculated as the time co ordinate t_{cg} corresponding to the centre of gravity of the curve and in the third method as the time t_m at which half the area of the curve has been recorded $t_{A/H}$ is supposed to represent the mean transit time through the monitored volume t_{cg} and t_m are supposed to represent the mean transit time from the site of injection and through half the blood volume in the monitored volume It has been shown, both theoretically and by studies on hypothetical model systems, that t_m in particular, constitutes a good approximation of the true mean transit time

CHAPTER 5

Material and methods

A Material

The experience from the technique presented in this study has been gained from about 40 experiments on dogs with various detectors and detector positions 21 experiments will be reported more or less in detail For these experiments the dogs were selected to a certain extent with regard to breed (the schafer and harrier were preferred) weight (amounting to 22 ± 5 kg)[1] and nutritional state

The animals were fasted for 24 hours before the experiment but had free access to water Anaesthesia was induced with Pentothal sodium® (Abbot Laboratories Ltd Queenborough Kent Great Britain) in individual dosage followed by chlora lose 1 % in saline (Chloralos puriss E Merck AG Darmstadt West Germany) in a dose of about 70 mg/kg initially, and smaller additional doses for maintenance of anaesthesia during the course of the experiment A tracheal tube was inserted to ensure a free airway During the actual experiment the dogs were placed on a slightly warm heat cushion

B Methods

a Operation

Calheters were introduced into a cubital vein for infusion of solutions, into one of the femoral arteries for blood pressure measurements and into both ureters for collec tion of urine A fine bore catheter was introduced via one of the femoral veins and placed with its tip in the inferior vena cava at the point of departure of the renal vein for recirculation studies

Through an incision in the left flank and careful retroperitoneal dissection a lumbar artery located opposite the point of departure of the renal artery from the aorta was exposed A mandrin supplied catheter with an external diameter of 0 75 mm (in early experiments a 1 02 mm catheter was used) was introduced through the lumbar artery and fixed with a loose ligature, after which it was passed through the aorta and into the renal artery With this technique no free dissection of the renal artery or hilus region was necessary In the majority of experiments the urine production was undisturbed during the catheterization

The kidney was then exposed through a peritoneal incision The detectors were introduced perpendicularly to the surface of the kidney with their tips oriented to wards the tip of the papilla The operation wound was then partially closed The remaining opening was covered with a plastic sheet to prevent drying Apart from a

The values in this chapter are given as mean±standard deviation

[1] Nylon intravenous cannula Cat No B205 Portland Plastics Ltd Hythe Kent Great Britain

slight luxation the kidney could usually be kept in its natural position In order to check its position Lissamin green was injected into the catheter If the position was correct the dye was distributed uniformly in the cortex corticis (a visually recorded indicator dilution curve) and excreted unilaterally in the urine At the same time the delay in the urine collection system was determined

b Protection from radioactivity

The treatment of the labelled red cells including washing pipetting etc , was carried out manually behind a lead glass window

Over the operation wound where the injection syringe with the radioactive blood was kept during the experiment (to keep it warm), a 60 cm wide and 10 mm thick Perspex plate was placed bent at an angle over the wound and supplied with rubber curtains 5 mm thick along the sides The radiation which amounted to between 100 and 500 mr/h (milliRontgen per hour) over the operation wound was thereby reduced to about 2 mr/h outside the Perspex plate and to about 20 mr/h outside the rubber curtains

c Performance of the experiments

0 2—0 7 ml of the labelled blood was injected as a slug into the renal artery The print out units were previously switched over to a print out rate of 2 readings per second in the one channel and in the decimal pip channel the paper speed of the recorder was increased to a rate allowing 5—10 readings per second The cortical recordings were made during about 2 min and the medullary recordings during about 5 min or more

For recirculation studies a usually larger amount (1—2 ml) of the labelled blood was injected into the fine bore catheter with its tip at the point of departure of the renal vein from the inferior vena cava

After about 10 minutes when the labelled red cells were assumed to be equilibrated in the total blood volume the background activity was determined during several 1 minute periods At this time point 5 ml blood were withdrawn via the catheter in the femoral artery and collected into small heparinized plastic cups

After completion of the experiment the same detectors that were used in vivo were immersed in the blood sample, after which the counting rate was determined during repeated 1 minute intervals The volume in the plastic cups exceeded the monitored volume which is essential for a correct determination of the regional red cell volume

d Calculations

In experiments including both intraarterial and intravenous injections the central recirculation curve was subtracted from the original curve where the recirculation curve was advanced 3 sec in time which was considered to correspond to the transit time through the renal artery and out into the inferior vena cava Obviously

it was not possible to determine the amount of injected radioactivity with complete precision In this way the recirculation curve at equilibrium could be somewhat higher or somewhat lower than the original curve even when the same amount was believed to have been injected into the renal artery as into the renal vein Since however the counting rate both in the original curve and the recirculation curve had reached a relatively constant value after 5 min the recirculation curve was adjusted so that the counting rate at 5 min was equal to that of the original curve

The curve thus obtained was then divided into two parts by semilogarithmic extrapolation of the descending part of the first part of the curve The area of the first part of the curve then comprised from about 70 % to almost 100 % of the total curve

This first part of the curve could be separated from the total curve without previous subtraction of the recirculation part for curves obtained in the cortex and in the medulla up to 10—12 mm from the cortico medullary border, since the central recirculation did not influence the first part of the curves at these locations of the detector

From the first part of the curve the mean transit time was calculated as $t_{A/H}$, t_{cg} and t_m where $\bar{t}_{A/H}$ was determined by dividing the area (counts) of the first part of the curve by the height of the rounded peak (cps) t_{cg} as according to equation 3 on p 27 and t_m as the time up to the point when half the area (half the number of counts) had been recorded

The red cell volume was calculated as the arterial haematocrit multiplied by the ratio between the counting rate recorded from a detector *in vivo* and that recorded from the same detector immersed in blood drawn from the animal

The total renal blood flow was calculated as according to equation 13 on p 30

e Controls general

The *rectal temperature* was measured in about half of the experiments and was found to be between 37 and 39 C

The *systemic blood pressure* was determined with a simple mercury manometer The mean pressure at the beginning of the experiments was found to be 123±12 mm Hg (n=21) During the course of the experiment the blood pressure remained relatively constant or possibly rose slightly

The *arterial haematocrit* was determined by centrifugation at 10 000 g in a micro capillary centrifuge (Model MB International Equipment Co Boston Mass USA) and was found to be 0 43±0 05 (n=18) No correction for trapped plasma was made as this only amounts to 1 3 % of the red cell column (Garby and Vuille 1961) During the course of the experiment the haematocrit remained essentially unchanged

The *acid base balance* was measured according to Siggaard Andersen with an Astrup micro equipment (Radiometer Copenhagen Denmark) For a detailed description including the theory normal values sources of error etc, see Siggaard Andersen (1964) Because of the contamination problem all acid base balance studies were carried out before any radioactive substance had been injected Since however

the duration of the experiments was only 1—2 hours and since both the respiration and blood pressure remained constant it is a reasonable assumption that the acid base balance during the actual experiment lay close to the control values The pH was found to be 7 39±0 04 (n=9), the P_{CO_2} 40±8 mm Hg (n=8) and the base excess — 0 7±4 5 mEq (n=8) The acid base status was thus fairly normal

f Controls, kidney function

The *urine production* was measured as drops with a simple drop counter and was recorded as pips with a photokymograph For more precise measurements, measuring cylinders were used At the beginning of the experiment the urine production was found to be 0 33±0 17 (n=21) ml/min from the right kidney and 0 40±0 22 (n=21) from the left kidney

The *urine osmolality* was determined by a freezing point depression method (Osmometer Model 31 LS Advanced Instruments Inc, Newton Highlands 61 Mass USA) For operating principles errors etc see Abele (1963) The osmolality of the urine from the right kidney was 1025±404 (n=17) mOsm/kg and of the urine from the left kidney 824±351 (n=17) mOsm/kg It was clear that the urine osmolality in the investigated left kidney was lower than that in the right control kidney which indicates some disturbance of the investigated kidney despite the relatively gentle technique The urine production was also greater on the left side

The *glomerular filtration rate* was determined as creatinine clearance For this 5 % creatinine was continuously infused at a rate sufficient to raise the plasma concentration to between 10 and 20 mg % As a rule urine was collected during a period of 10 minutes In the middle of this period (correction was made for the delay in the urine collection system) blood samples were taken Creatinine in the urine and plasma were analysed according to Bonsnes and Tausky (1945) The glomerular filtration in the right kidney was found to be 0 76±0 25 (n=19) ml/min and gram[1] tissue and in the left kidney 0 74±0 20 (n=19) ml/min and gram tissue During the course of the experiment the creatinine clearance usually fell somewhat

In addition to these control studies routine examinations for PAH clearance urine conductance and the concentrations of sodium and potassium in the urine were performed These have not been treated systematically however

[1] The weight refers to kidney weighed without previous ligation of the renal pedicle On correction for a blood loss of 30 (Swann 1960) a value of 0 53 ml/min and gram tissue was obtained

CHAPTER 6

The regional circulatory pattern in the kidney

A Methodological aspects

The regional circulatory pattern in the kidney was determined from 63 detector positions in 7 dogs weighing between 15 and 32 kg, and with a mean weight of 22 kg In this series only those experiments were included, in which acceptable indicator dilution curves were obtained from at least 4 different detector positions in the renal parenchyma

Two detectors usually ND 1 5 and ND 2 0 ,were first placed deeply into the inner zone with their tips directed towards the tip of the papilla After one or more injections into the renal artery, the detectors were then withdrawn in 3—5 mm stages The advantage of this technique was that in this way the non perfused zone contained inactive blood at all positions of the detector whereas if the detector had been moved from more superficial to deeper positions then it would have damaged the tissue containing active blood which would then have been included in the non perfused zone and would have given rise to an unfavourably high background level

In order to investigate possible changes in the regional blood flow during the course of the experiment one detector was held in a fixed position in the cortex in two experiments Values of 1 1 1 3 and 1 2 sec were then obtained for t_m in one experiment and 1 3 ± 0 1 (n=5)[1] in the other In dog No 7 a detector ND 1 5 was placed deeply into the inner zone of the medulla and was then moved outwards to more superficial positions while another detector ND 2 0 was first placed superficially and then moved to deeper positions The results of this study are shown in Table 2 The table can at the same time give an idea of how the experiments reported here are conducted It can be concluded that the changes in the regional blood flow during the course of the experiment were fairly moderate

B The regional circulatory pattern in the cortical parenchyma

a Shape of the cortical indicator dilution curves

Indicator dilution curves obtained from detector positions in the cortical parenchyma are illustrated in Figs 9 to 12 Fig 9 shows a curve obtained with a detector placed about 1 mm from the cortico medullary border There is a biphasic appearance typical of this detector position with a small initial peak and a broad shoulder When the detector is moved to a position 3 mm from the cortico medullary border the biphasic appearance becomes less pronounced but can still be clearly observed (Fig 10) In curves obtained from detector positions in the middle of the cortex (Fig 11) the first sharp peak is hardly distinguishable and at detector positions in the more superficial parts of the cortex (Fig 12) it has disappeared completely

[1] Mean $\pm$standard deviation

TABLE 2 Data obtained from one experiment in which one detector (ND 1 5) was first placed other detector (ND 2 0) was first placed superficially and then moved to deeper positions studies are carried out with intravenous injection of labelled red cells into the inferior

Dog 7 Schafer
Weight 32 kg Left kidney weight 97 g Acid base status pH 7 37 P_{CO} 43 mm Hg Base excess 0 mEq

Injection	Detector	Region	Position mm from CM border	t_{peak} sec	$\bar{t}_{A/H}$ sec	t_{cg} sec
1	ND 1 5	Inner medulla	12 0	8 0	33 2	26 6
	ND 2 0	Outer medulla	4 0	2 0	6 5	5 6
2	ND 1 5	Inner medulla	12 0	—	—	—
	ND 2 0	Outer medulla	4 0	—	—	—
3	ND 1 5	Inner medulla	7 5	5 0	9 0	7 9
	ND 2 0	Inner medulla	7 5	5 8	10 7	10 7
4	ND 1 5	Inner medulla	7 5	—	—	—
	ND 2 0	Inner medulla	7 5	—	—	—
5	ND 1 5	Outer medulla	4 0	1 3	5 5	5 5
	ND 2 0	Inner medulla	12 5	8 0	21 6	21 0

In spite of the different appearances of the cortical indicator dilution curves, the time course expressed as mean transit time is relatively similar[1] at all levels in the cortex (see Figs 13 and 14) Assuming that this is true the calculated mean transit times were as follows $t_m = 1\ 3 \pm 0\ 2$ sec $\bar{t}_{cg} = 1\ 6 \pm 0\ 3$ sec and $t_{A/H} = 2\ 1 \pm 0\ 4$ sec

b Interpretation of the cortical indicator dilution curves

It seems probable that the first sharp peak which is especially prominent in the indicator dilution curves obtained from detector positions near the cortico medullary border was generated by the indicator during its passage in the arcuate arteries and the proximal parts of the interlobular arteries The subsequent more extended wave was probably generated from the indicator in the glomerular and peritubular capillary systems

Because of this it follows that the initial peak is less pronounced in curves obtained when the detector is in the middle of or on the surface of the cortex since the arcuate arteries are then included in the periphery of the monitored volume With the detector in the superficial part of the cortex the curve should reflect almost exclusively the passage of the indicator through glomerular and peritubular capillaries

With regard to the calculated mean transit times it can be stated that the pre

[1] Reservation will be made here for the transit time in the outer layer of the cortex In some studies where a needle detector was placed in the middle of the cortex and an end window detector on the surface of the cortex a shorter transit time was obtained when calculated from the indicator dilution curve of the surface detector

deep into the inner zone of the medulla and was then moved to more superficial positions and the C_{PAH} and $C_{Creatinine}$ denote clearance of PAH and creatinine respectively The recirculation vena cava at the point of departure of the renal vein

$\bar{t}_m$ sec	Red cell volume	Arterial pressure mm Hg	Urine flow right ml/min	Urine flow left ml/min	Comments
21 6	3 3	115	0 4	0 3	C_{PAH} (ml/min) right 258
4 2	8 8				left 244
					$C_{Creatinine}$ (ml/min)
					right 72 left 78
—		115	0 41	0 33	Recirculation study
—					
6 9	6 6	115	0 4	0 3	
8 7	6 3				
—		115	0 4	0 3	Recirculation study
—					
3 6	6 7	115	0 4	0 3	
16 6	—				

requisites for correct determination of these as t_m (see chapter 4) were good Mean transit times calculated as $t_{A/H}$ were more uncertain It seems not improbable, however that at the time when the peak of the second wave was recorded the indicator was fairly evenly distributed within the monitored volume In calculating the mean transit time as $t_{A/H}$ therefore the height of the second wave was used

c Calculations

As discussed previously when calculating the blood flow in an organ or part of an organ the relationship

$$V = F \times t$$

can be used where V is the blood volume F the blood flow and t the mean transit time for blood particles In the present investigation labelled red cells were used as the indicator and therefore the mean transit time refers primarily to the mean time for red cells to pass through the red cell volume in a region By dividing the red cell volume by the calculated mean transit time the red cell flow is obtained

Mean transit time

As discussed above though the appearance of indicator dilution curves obtained from different regions of the cortical parenchyma differed from each other, the mean transit time was approximately equal at all levels This means that the mean transit

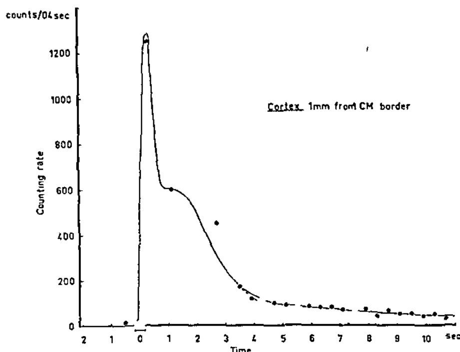


Fig 9 Cortical indicator dilution curve obtained from a detector placed 1 mm from the cortico-medullary border (dog No 5) → denotes the time of injection of the P³² labelled red cells into the renal artery The experimental values correspond to the number of counts recorded during a time interval of 0 4 sec The continuous line is drawn to the best visual fit The broken line represents a semilogarithmic extrapolation of the first part of the curve Note the biphasic appearance with a narrow peak followed by a broad sholder The first peak is probably generated by the indicator during its transit through the arcuate arteries (arcuate peak)

time calculated from a curve obtained from a detector position in the middle of the cortex is representative of the cortex as a whole

Three different methods of determining the transit time have been discussed previously (see chapter 4) It was found that the time t_m at which half the area of the indicator dilution curve had been recorded constituted a good approximation of the value for the true mean transit time from the site of injection and through half the monitored blood volume i e in this case that time required for the labelled red cells to pass from the injection site in the renal artery and through half the red cell volume in the cortical parenchyma As the blood volume in arteries and veins below the cortex (including the arcuate arteries and the arcuate veins) is essentially the same (Weaver et al 1956) the mean transit time from the renal artery through the cortical parenchyma and out into the renal vein is obtained as 2 $\bar{t}_m$ For determination of the mean transit time through the cortical parenchyma itself $\bar{t}_m$ must be corrected for the time required for the red cells to pass from the renal artery to the cortex In the present study this time was estimated to be about 0 3 seconds from the first narrow arcuate peak in those indicator dilution curves which were

counts/0.4sec

Counting rate

1000
800
600
400
200
0

Cort x. 3mm from CM-border

1 0 1 2 3 4 5 6 7 8 9 10 11 sec

Time

Fig 10 Cortical indicator dilution curve recorded from a needle detector placed 3 mm from the cortico medullary border (dog No 5) The biphasic appearance is now less pronounced then in Fig 9 because the arcuate arteries are locate 1 m the periphery of the monitored volume

counts/0.4 sec

Counting rate

700
600
500
400
300
200
100
0

Cortex 5mm from CM–border

1 0 1 2 3 4 5 6 7 8 9 10 11 sec

Time

Fig 11 Cortical ind cator dilution curve recorded from a needle detector placed 5 mm from the cortico-medullary border (dog No 1) The biphasic appearance is now hardly distinguishable because the arcuate arteries are located outside the monitored volume

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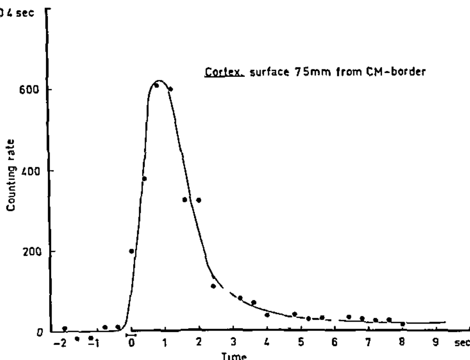


Fig 12 Cortical indicator dilution curve recorded from an end window detector placed on the surface of the kidney (dog No 1) At these detector positions the curves have a fairly symmetrical appearance

obtained from detector positions in the vicinity of the cortico medullary border According to Weaver et al (1956) 2 4 % of the distended kidney weight is to be found in the arteries below the cortex Assuming a blood flow of 4 ml per minute and gram tissue in the kidney the transit time of blood in these arteries would be 0 36 sec If it is taken into account that in this case the catheter was inserted 2—3 cm into the renal artery and that the transit time is less for red cells it is not improbable that this value also will be about 0 3 sec

In this series of experiments $\bar{t}_m$ in the cortex averaged 1 3 sec The transit time from the renal artery through the cortex and out into the renal vein will then be 2 1 3=2 6 sec and the transit time through the cortex itself will be 2 (1 3—0 3) = =2 0 sec The latter value agrees well with the mean transit time of 2 1 sec through the monitored volume calculated as $\bar{t}_{A/H}$

The mean transit times calculated as $\bar{t}_{cg}$ were about 20 % greater than $\bar{t}_m$

Red cell volume

The cortical red cell volume was determined according to the method described on pp 42 and 43 In this parenchyma, however, the method was found to be less suitable due to the relatively large size and size variations of the non perfused zone' in the fragile cortical parenchyma Haemorrhages were often seen around the detector

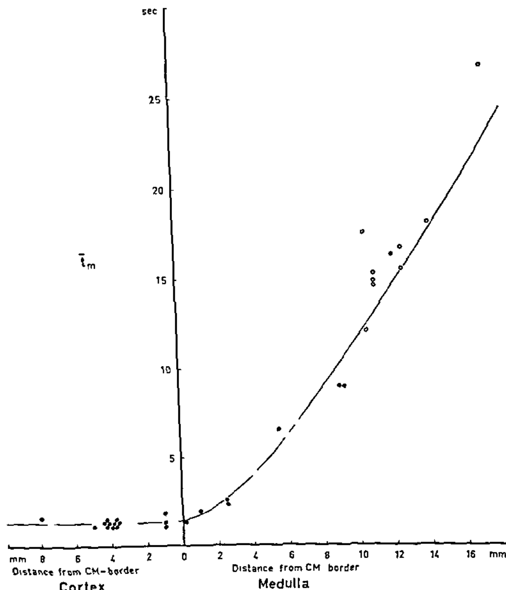


Fig 13 Data obtained from 63 different detector positions in 7 dogs on $\bar{t}_m$ as calculated from the first part of the indicator dilution curves Open circles denote values obtained from detector positions more than 10 mm from the cortico-medullary border in which the semilogarithmic extrapolation of the first part of the curves has been performed without subtraction of the central recirculation via the heart (for explanation see text) The curve is drawn to the best visual fit

In the cortex t_m is roughly the same at all levels within the parenchyma averaging 1 3 sec in this series In the medulla $\bar{t}_m$ increases from about ? 5 sec in the middle of the 4—6 mm thick outer zon to about half a minute near the tip of the papilla

The cortical red cell volume has been determined by several authors previously by different methods Table 3 summarizes some of the more important of these investigations with regard to regional and total red cell volumes in the dog

Table 3 *Data of total and regional red cell volume in the dog kidney obtained from the literature* and the present investigation Values given in round numbers denote that these have been calculated or estimated from original data

Authors	Method	Red cell volume whole kidney			
Lilienfield et al 1957	From flow and mean transit time	8 4 %			
Ochwadt 1957	From flow and mean transit time	8 3 %			
Polosa and Hamilton 1962	Drainage washout technique	7 3 %			
Polosa and Hamilton 1963	Drainage washout technique	8 %			
		Regional and red cell volume			
		Cortex	Outer zone	Inner zone	Papilla
Lilienfield et al 1958	Cr^{51} labelled red cells	6 9 %	10 4 %	6 3 %	3 5
Emery et al 1959	P labelled red cells	8 %	6 % *	6 %	6—9 %
Ullrich et al 1961 a	Haemoglobin content	9 65 %	10 50 %	3 75 %	
Wolgast 1968	P labelled red cells and internal monitoring	—	7 5 % *	6 3	4—12%

*The transitional zone excluded

A few comments are pertinent with regard to the values in this table The red cell volume calculated from flow and mean transit time, and also that calculated by the drainage washout technique refer mainly to the red cell volume in the entire kidney, including afferent and efferent vessels Since the cortical tissue volume comprises approximately 70 % of the entire renal volume (von Mollendorff and Schroder 1930) and in addition the red cell volumes in all regions of the kidney are relatively equal the values for the red cell volume per gram tissue of the whole kidney may also be considered to apply to the red cell volume in the cortex including most of the volume in afferent and efferent vessels as expressed per gram of the cortical tissue

The regional red cell volume determinations refer to the red cell volume in the parenchyma itself but *it cannot be excluded that certain larger vessels, e g the* arcuate arteries and veins are included in certain investigations

It would seem from the table that the data concerning the cortical red cell volume are in relatively good agreement However differentiation between the purely intracortical red cell volume on the one hand and the intracortical red cell volume plus that in afferent and efferent vessels on the other, seems difficult

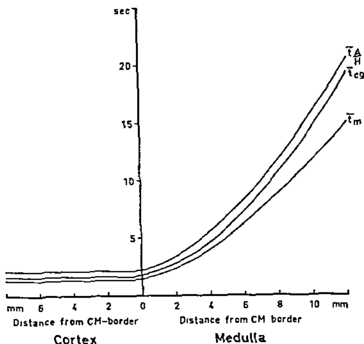


Fig 14 Comparison between $\bar{t}_m$ t_{cg} and $\bar{t}_{A/H}$ calculated from the first part of the indicator dilution curves obtained from different detector positions in cortex and medulla

The curves are drawn to the best visual fit data obtained from the same experiments as in Fig 13 The curves are similar in shape with the exception that the $\bar{t}_{A/H}$ values are higher than the corresponding t_{cg} values which in turn exceed the $\bar{t}_m$ values

Cortical blood flow

If the mean transit time is determined as 2 t_m and assuming an undifferentiated red cell volume of 8 % the cortical red cell flow is calculated as 1 8 ml/min and gram tissue If on the other hand the mean transit time is determined as 2 corrected t_m the red cell flow is calculated as 2 4 ml/min and gram tissue Using $t_{A/H}$ the red cell flow will be 2 3 ml/min and gram tissue A value of 2 ml/min and gram tissue then seems to be a good approximation of the cortical red cell flow By dividing this value by the arterial haematocrit in this series 0 44 the cortical blood flow is obtained as about 4 5 ml/min and gram tissue

In a previous investigation (Aukland and Wolgast 1968) the cortical flow calculated by this indicator dilution method was compared with the flow calculated from the total flow measured by an electromagnetic flow meter and the weight of the cortex The transit time was determined as 2 corrected t_m and the regional red cell volume was assumed to be 8 % The cortical weight was assumed to constitute 70 % of the total kidney weight From the results given in Fig 15 the conclusion can be drawn that the blood flows calculated from the two methods do not differ essentially

This comparison shows that the blood flow in the monitored volume ou

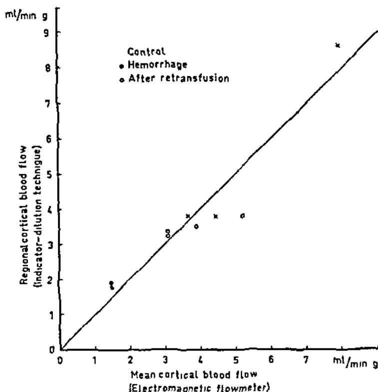


Fig 15 Comparison between regional cortical blood flow calculated from total renal blood flow (electromagnetic flow meter) and weight of the cortical parenchyma (abscissa) and by the indicator dilution technique (ordinate) The straight line is the line of identity
Modified from Aukland and Wolgast (1968)

non perfused zone is obviously equal to the regional blood flow in the rest of the cortex i e *the regional blood flow in the monitored volume is not seriously disturbed by the detector and puncture trauma*

C *The regional circulatory pattern in the medullary parenchyma*

a Shape of the medullary indicator dilution curves

The appearances of indicator dilution curves obtained from detector positions at different levels in the renal medulla are shown in Figs 16—20 Fig 16 shows an indicator dilution curve obtained from a detector position about 1 mm from the cortico medullary border The profile is typical for this position with a distinctly delimitable narrow initial peak followed by a second wave more extended in time The second wave falls approximately monoexponentially down to about 5 seconds after which it changes over to a more slowly descending course At detector positions 3 mm from the cortico medullary border (Fig 17) the initial rapid peak can still be observed clearly Apart from the fact that in this position the curve is more extended in time

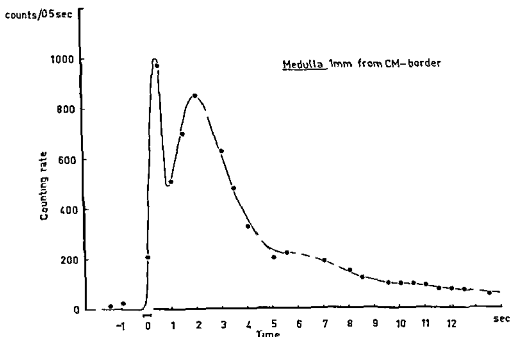


Fig 16 Outer medullary indicator dilution curve obtained from a needle detector placed 1 mm from the cortico medullary border (dog No 5) For this detector position the curve has a typical biphasic appearance with an easily distinguishable narrow arcuate peak followed by a second wave more extended in time

the appearance is essentially the same At detector positions 4 mm from the cortico medullary border (Fig 18 a) the initial peak has practically disappeared The descending part of the curve deviates from its monoexponentially descending course after about 7 sec This deviation coincides approximately in time with the central recirculation via the heart (the thinner unbroken line) The original curve and the recirculation curve do not attain the same value however until after almost 2 min Fig 19 illustrates a curve from a detector placed 7 5 mm from the cortico medullary border i e in the inner zone of the medulla The curve has acquired a biphasic appearance here by deviating from its monoexponentially descending course relatively early and changing into a more slowly falling or even an initially somewhat ascending course At detector positions deep in the inner zone (Fig 20) the appearances of the curves are more reminiscent of those obtained from detector positions in the outer zone The central recirculation here occurs early in the course of the curve and influences the initial descending part of the curve

The time courses of the curves expressed for example, as $\bar{t}_m$ vary from about 2 5 sec in the middle of the outer zone to about half a minute down towards the tip of the papilla (Fig 13)

The pattern of the mean transit time in the medulla thus differs completely from that in the cortex

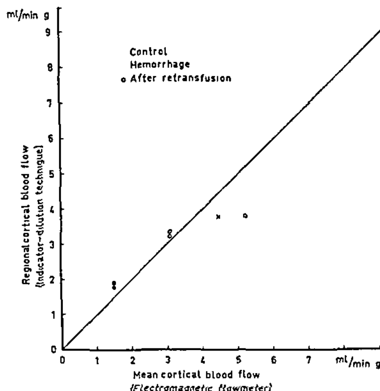


Fig 15 Comparison between regional cortical blood flow calculated from total renal blood flow (electromagnetic flow meter) and weight of the cortical parenchyma (abscissa) and by the indicator dilution technique (ordinate) The straight line is the line of identity
Modified from Aukland and Wolgast (1968)

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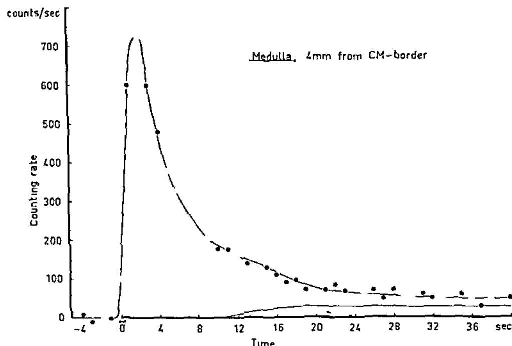


Fig 18a Outer medullary indicator dilution curve obtained from a needl detector placed 4 mm from the cortico medullary border (dog No 7) The thinner continuous line indicates the curve for central recirculation i e recirculation via the heart

After subtraction of the central recirculation the curve can be divided into two parts by semi logarithmic extrapolation of its first part The size and appearance of the first part are evident from the figure This part is assumed to arise from the indicator during its first transit through the monitored volume

The second part is obtained by subtraction of the first part from the total curve and is shown in figure 18b

to the fraction of the total amount of radioactivity perfusing the pathway but rather to the blood volume in the pathway (see eq 10 on p 29) At detector positions in the middle of the outer zone the arcuate peak is relatively less prominent because the region around the cortico medullary border lies in the periphery of the monitored volume The prominent feature of all curves obtained from the outer zone is that the curves fall monoexponentially during the greater part of their courses and then change over relatively markedly to a very slowly descending part A part, but not the whole of this tail is caused by the central recirculation as is clearly evident in Fig 18 a

After subtraction of the central recirculation the curve can be divided into two parts by semilogarithmic extrapolation of the first part of the curve shown in the figure as the dashed line

The appearance and size of the first part, part 1 is directly evident in Fig 18 a The second part part 2 is shown on an enlarged scale in Fig 18 b It is probable that

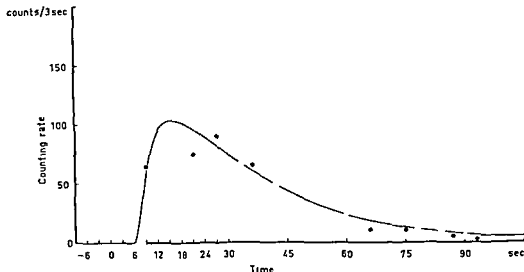


Fig 18b Second part of the indicator dilution curve in Fig 18a shown on an enlarged scale This curve is assumed to arise from the indicator which has returned from capillary systems located below the monitored volume i e from the inner zone of the medulla and now passes through the monitored volume in ascending vasa recta

counts/sec

M.dulla, 7.5mm from CM-border

Fig 19 Inner medullary indicator dilution curve obtained from a needle detector placed 7.5 mm from the cortico medullary border (dog No 7) The thinner continuous line is the curve for the central recirculation The curve is divided into two parts by semilogarithmic extrapolation of its first part The size and appearance of the first part of the curve are clearly evident from the figure The second part is made up of the area between the total curve minus the central recirculation and the first part of the curve

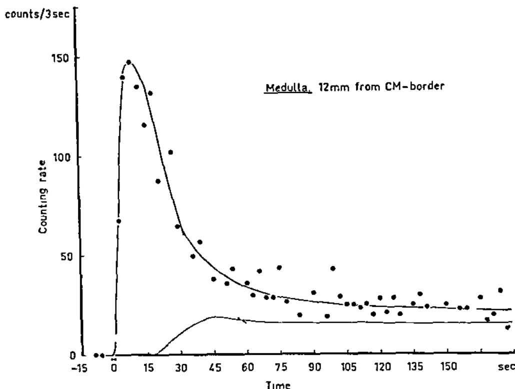


Fig 20 Inner medullary indicator dilution curve obtained from a needle detector placed 12 mm from the cortico medullary border (dog No 7) The time lapse of the extrapolated first part of the curve is now about one minute It is evident from the figure that the central recirculation (the continuous line) will influence the descending part of the first part of the curve This influence will cause an error in the mean transit time determination of the first part of the curve of 10—30 , in curves obtained from detector positions more than 10 mm from the cortico medullary border if this is calculated without previous subtraction of the central recirculation

the first part is generated by the indicator during its first passage through the monitored volume Part 2 is then generated by the indicator which has returned from capillary systems situated below the monitored volume and which now passes into the ascending vasa recta through the monitored volume, but it is also generated to a small extent by free P^{32} phosphate Part 2 is thus generated to the largest part by the '*internally recirculating indicator* It may be pointed out here that extrapolation of the first part of the curve is a graphic construction and that this construction does not perhaps reflect sharply the postulated circulatory event As discussed in chapter 4 a moderately erroneous extrapolation introduces on the other hand relatively little error in the final blood flow calculations

In order to make this detailed analysis of the indicator dilution curve it is important that the relative amount of free P^{32} phosphate or other low molecular P^{32} compounds in the injected bolus is very small In this experiment as in the

experiments behind the curves in Figs 19 and 20 the P^{32} labelled red cells were therefore washed immediately before the injection A similar division into two parts can be made in curves obtained from the inner zone of the medulla, as can be seen from Fig 19

The number of recorded counts from part 2 in relation to the whole indicator dilution curve was found to be the same in general in curves obtained from the outer zone as in those from the outer parts of the inner zone In curves obtained from the papillary region part 2 appears to be relatively smaller From 7 determinations on 6 dogs part 2 was calculated to comprise about 30 % of the total curve (range 20—36 %) in the outer zone and the outer parts of the inner zone

As has already been pointed out, this 30 % arises from the internally recirculating labelled red cells but also partly from free P^{32} phosphate or other low molecular P^{32} compounds

The fraction of the counts arising from low molecular P^{32} compounds is not necessarily proportional to the fraction of these compounds in the injected slug since the number of counts arising from an indicator is proportional to its volume of distribution rather than the fraction of the indicator perfusing the region in question (see eq 10 on p 29) In the cortex which is considered to constitute a homogeneously perfused parenchyma the indicator dilution curves in spite of this end in a more or less pronounced tail which to a great extent arises from free P^{32}-compounds If the labelled red cells are washed immediately before the injection this tail is small (see Fig 12 p 50 and Fig 26 p 71) but may constitute up to about 10 % of the total curve

In the medulla and especially in the inner zone the part arising from free P^{32} phosphate is probably less since low molecular compounds will be shunted between the vasa recta limbs Assuming that 5 % had arisen from free P^{32} compounds then 25 % of the total number of recorded counts would arise from the recirculating red cells and 75 % from the labelled cells during their first transit through the monitored volume The transit time which is calculated from part 1 of the curve then refers to the transit time through 75 % of the red cell volume in the monitored volume (for further explanation see chapter 4 especially p 39)

c Calculations

Detected regions

In the calculations of the blood flow in the renal cortex it was established that the mean transit time was in general the same in all regions of the cortical parenchyma In the renal medulla the conditions were completely different The mean transit time calculated somewhere in the inner zone is thus not representative of the mean transit time in the whole inner zone It is thus necessary to define for what region in the medulla the determined mean transit time is representative One seemingly satisfactory way of treating this question can be seen in Fig 21 The figure shows a kidney cut in two planes at right angles to each another The cortex is indicated by the shaded surface and the medulla as the unshaded surface The medulla is divided

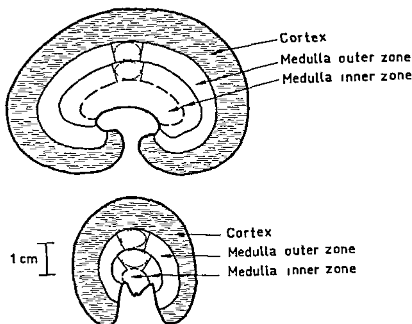


Fig 21 Kidney cut in two planes at right angles to each other (Drawn from photographs) The medulla is divided into the outer zone the 5—6 mm thick outer part of the inner zone and the papillary region Two needle detectors are placed in the middle of the first and second layers The monitored volume is indicated as the 1 % iso counting rate line It is evident that the needle detectors can be considered to monitor the volume of the sectors given in the figure

into three layers The first layer is made up of the 4—6 mm thick outer zone seen in these series In the kidney illustrated in Fig 21 the outer zone was 6 mm The second layer comprises the 5—6 mm thick outer part of the inner zone and is thus delimited by the borderline between the outer and inner zones on one side and an imaginary plane cut perpendicular to the strial direction in the region and situated 5—6 mm from the border between the outer and inner zone on the other side The third layer comprises the papillary region

Needle detectors are placed in the middle of the first and second layers The monitored volume is indicated here by the 1 % iso counting rate line (see p 23) The extent of the monitored volume in the longitudinal axis of the detector is then 5 5 mm It would seem evident from the figure that the needle detectors can be considered to monitor the volume of the sectors which are given in the figure. It is now probable that the mean transit time in such a sector is the same as in all sectors in the layer and thus also the same as the mean transit time in the layer as a whole This means that a detector placed in the middle of a 5—6 mm thick layer in the medulla monitors a volume which from the point of view of the mean transit time is representative of the layer in question

In this connection the monitored volume has been considered to have an extent of 5—6 mm in the longitudinal axis of the detector Under this assumption 90—95 %

of all counts will arise from the indicator in the layer Obviously it can also be assumed that the detector monitors a representative part of a 3 mm or perhaps 8 mm thick layer Under these circumstances, 70 % and 99 % respectively, of all counts will arise from the assumed layers If, as in the case of a 3 mm thick layer, only 70 % of all counts arise from the layer, it is doubtful whether the monitored volume is representative of the layer For the case of an 8 mm thick layer, it can be stated that this is 60 % thicker than the 5 mm layer, and that this increase only corresponds to a 9 % increase in the number of counts recorded from the layer The monitored volume can thus hardly be regarded as representative of a layer as thick as 8 mm For the final calculation of the blood flow in the medulla however, the use of slightly different layer thicknesses does not play a large role

Mean transit time

The possibilities were discussed in chapter 4 for determination of the mean transit time in the renal medulla It was established then that t_m and also $t_{A/H}$ constituted acceptable approximations for the mean transit time The aim of $t_{A/H}$ was to determine the mean transit time through the actual monitored volume while t_m should represent the mean transit time from the injection site and through half the blood volume in the monitored volume In order to determine the mean transit time in a layer when t_m is used it is then necessary to determine the time required for the indicator slug to pass from the site of injection in the renal artery to the upper border of the layer i e the transit time in the main arteries the juxtamedullary glomeruli and descending vasa recta up to the border of the layer It was found possible to determine this transit time from the indicator dilution curves obtained which will be discussed in the following

Determination of the linear rate in descending vasa recta

Fig 22 shows a simple circulatory system representing a capillary network[1] situated around the centre of the needle detector with afferent and efferent vessels corresponding to descending and ascending vasa recta To the right is shown a corresponding indicator dilution curve obtained after a slug injection of P^{32} labelled red cells into the renal artery It is assumed that the bolus is maintained as an ideal slug during the transit in the descending vas rectum At the time of the injection the radioactive indicator bolus will be outside the monitored volume and the detector will not record it At position (I) (see figure) the bolus passes into the monitored volume and the detector will record the indicator but now with low efficiency since it is in the periphery of the monitored volume As the indicator bolus approaches the centre of the detector the counting rate recorded by the detector increases to reach a maximum just when the indicator is located around the centre of the detector i e at the time point when the indicator bolus is just about to pass into the capillary network (II) During the passage of the indicator through the capillary network this maximal count rate is maintained for a long or short period When the indicator

[1] This capillary network can represent all the capillary networks around the centre of the detector

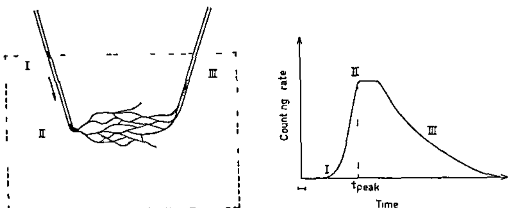


Fig 22 A medullary capillary system located in the central region of the mon tored volume (broken line) with its afferent and efferent vessels corresponding to descending and ascending vasa recta respectively The figure to the right shows the indicator dilution curve obtained after a slug injec tion into the renal artery When the bolus is just about to pass into the monitored volume (position I) the indicator will be recorded by the detector but now with low efficiency As the bolus approaches the centre of the monitored reg on the counting rate successively increases to reach a maximum when the bolus is just about to pass into the capillary system (position II) This maximal counting rate will be maintained as long as the entire bolus is located in the capillary system When the indicator subsequently passes out from the monitored volume the counting rate will decrease towards zero (position III)

subsequently passes out from the monitored volume the counting rate decreases toward zero (III)

It can thus be stated that the earliest time point at which the curve has reached its maximal value corresponds to the time at which the indicator in the descending vas rectum bound for the capillary systems around the centre of the detector has just reached these systems

If the indicator slug is extended in time, the curve will attain a more rounded peak, where the time point for this peak will correspond to that time when the entire indi cator slug has passed into the capillary system assuming obviously that none of the indicator at this time point has passed out from the capillary system The time point of the peak of the curve will in other words overestimate the mean time for the pas sage of the indicator through the descending vas rectum

We will now discuss the more realistic system with capillary systems located at all levels within the monitored volume In this system we *assume* that the time when the indicator dilution curve has reached its highest point t_{peak} corresponds to the time when the centre of gravity of the indicator bolus bound for the capillary systems around the centre of the detector has just reached these systems

Fig 23 shows the t_{peak} values determined in this series From this figure, the distribution of the indicator within the monitored volume at the assumed time point can be determined Fig 24 shows how the centres of gravity of the different indicator boluses are distributed within a monitored volume located with its centre 8 mm from

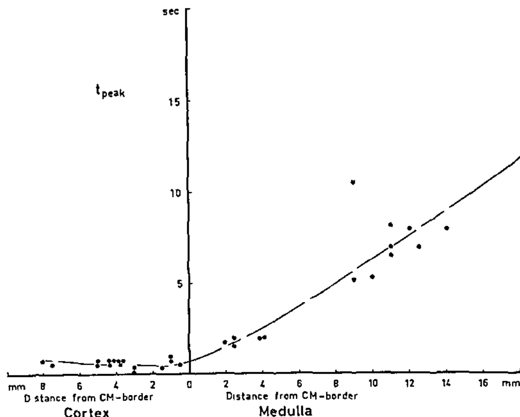


Fig 23 The figure summarizes the t_{peak} values obtained from the same experiments as outlined in Fig 13 The curve is drawn to the best visual fit In the medulla t_{peak} increases relatively linearly with the distance from the cortico-medullary border The curve cuts the t_{peak} axis with a time intercept of 0 7 sec

the cortico medullary border The indicator bound for the capillary systems located above the centre of the detector has now for the greatest part, passed into the respective capillary systems and is detected with an efficiency which for that part of the indicator is maximal or almost maximal The centre of gravity of the indicator boluses bound for capillary systems situated below the centre of the detector has now almost reached a level at the centre of the detector and in other words will be recorded with almost maximal efficiency The mean efficiency with which the whole of the indicator distributed in the way described is recorded is probably less than maximal i e the distribution is not compatible with recording of the peak of the curve The assumed time is then wrong

If the time sought is assumed to be half a second before t_{peak} the distribution of the indicator is roughly the same as in Fig 24 With an advance in time of half a second i e when the efficiency should be maximal the greatest part of the bolus bound for the central capillaries has passed into these and is detected with maximal efficiency The indicator bound for the capillaries located above the centre may now

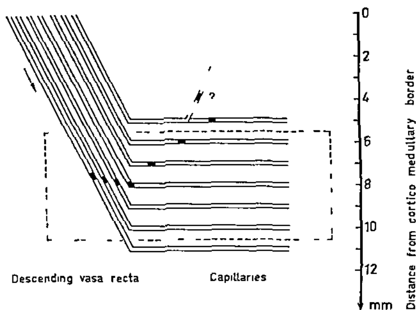


Fig 24 Distribution of the indicator in vasa recta and their corresponding capillary systems at that time point when the bolus bound for the capillary system located in the central region of the monitored volume (broken line) is just about to pass into the capillary system One capillary system is illustrated schematically as one vessel having the same diameter as the descending vasa recta The bolus bound for the capillary system located above the central region has now passed into the respective capillary systems and is detected with maximal efficiency If the capillary volume is small the bolus in some of the capillary systems may have passed into ascending vasa recta (the vessel indicated by the broken lines) and will then be detected with less than maximal efficiency The bolus bound for capillary systems located below the central region has now almost reached the central level of the monitored volume and will be detected with almost maximal efficiency

at least partly have left the capillaries and will be detected with lower efficiency The indicator boluses bound for the capillaries located below the centre of the detector have now passed the central level and are then detected with lower efficiency The mean efficiency of the total indicator is probably then not maximal which should be the case if the assumed time was correct

From this reasoning we may draw the conclusions that the assumed time t_{peak} will probably exceed the time point when the boluses bound for the capillary systems located around the centre of the detector have just reached these systems The error is however small and is probably less than half a second

For the evidence to be complete the same reasoning must be employed in principle for all other times on the curve If for example the time sought is assumed to be that time when the curve has reached half its maximal height the distribution of the indicator will also be approximately as in Fig 24 This distribution is not compatible with the fact that the curve has only reached half its maximal height In other words the assumed time is erroneous and so on

Without employing this reasoning for all times it can be stated that t_{peak} *corresponds well to the time sought*

It is possible now to obtain from the curve in Fig 24 quantitative data on the linear rate in the descending vasa recta It can be seen that the curve rises relatively linearly with the distance from the cortico medullary border The curve cuts the t_{peak} axis with a time intercept This intercept indicates that the transit time of the indicator from the renal artery through the juxtamedullary glomeruli and up to the beginning of the descending vasa recta, is of the order of about one second (0 7 sec), a value which seems not unreasonable The linear rate for red cells in descending vasa recta is now obtained by dividing their length by t_{peak} minus 0 7 sec

In descending vasa recta bound for the middle of the outer zone the linear rate was found to be 2 5 mm/sec, in descending vasa recta bound for the outer parts of the inner zone about 2 mm/sec and in descending vasa recta bound for the papillary region about 1 5 mm/sec

It is now possible to calculate the mean transit times in the different layers A summary of these calculations is given in Table 4 The outer zone corresponds to the approximately 5 mm thick dark red outer part of the renal medulla seen in this series The outer part of the inner zone is defined as the 5—6 mm thick layer in the outer part of the inner zone The papillary region is defined as the region between about 11 mm from the cortico medullary border and the tip of the papilla

It is evident from the table that the mean transit times calculated from t_m and t_{peak} show approximately the same value as $t_{A/H}$ The values calculated from t_{cg} and t_{peak} are somewhat higher, although the differences must be regarded as moderate

Red cell volume

The regional red cell volume in the medulla was determined by the method described in chapter 5 pp 42 and 43 The results from 11 dogs in this series and autoregulation series (see chapter 7) are presented in Fig 25 In this presentation only those results have been included which were obtained during the first half hour of each experiment The values from the later parts of the experiments do not differ essentially however from these early values

Near to the cortico medullary border the red cell volume appears to be relatively large, but the experiments are too few to draw any definite conclusions This large

TABLE 4 Mean transit time for red cells in the different medullary regions

	$t_{A/H}$	t_m and t_{peak}	t_{cg} and t_{peak}
Outer zone	3 9 sec	4 0 sec	5 2 sec
Inner zone (outer 5—6 mm thick part)	11 sec	11 sec	15 sec
Inner zone (papillary region)	about 30 sec	about 30 sec	about 30 sec

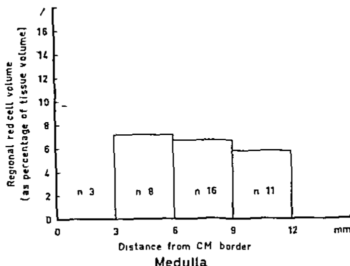


Fig 25 Regional red cell volume in the medulla up to 12 mm from the cortico-medullary border expressed in per cent of tissue volume Values obtained from detector position between 0 and 3 mm 3 and 6 mm etc are grouped together The values obtained from positions more than 12 mm from the cortico medullary border are few and scattered

Assuming the red cell volume to be linearly related to the distance from the cortico medullary border the standard error of estimate was found to be 1 3 % as calculated by the method of least squares

volume can probably be ascribed to the fact that the richly vascular region in the border layer between cortex and medulla is included in the monitored volume At the more certain determinations from detector positions 3—6 mm from the cortico medullary border the red cell volume was 7 2 % In the middle of the 5 mm thick outer zone in this material, this value was 7 5 % In the inner zone up to 12 mm from the cortico medullary border a value of about 6 % was obtained The red cell volume determinations in the papillary region are few and show varying results (4—11 %)

Assuming the red cell volume to be linearly related to the distance from the cortico medullary border the standard error of estimate was found to be 1 3 % as calculated by the method of least squares This corresponds to a coefficient of variation of 15—20 % thus a rather great variance For comparison it may be mentioned that Lilienfield et al (1958) found a coefficient of variation of 10—15 % Assuming that small methodological errors are inherent in the method of Lilienfield et al, this would mean that the biological variation is of the order of 10 % and that the variation due to methodological error in the present method would be about 5—10% As some extreme values which probably were caused by instability of the detecting system[1] are included in the series presented the methodological error is probably overestimated somewhat

[1] The detectors have been improved continuously Some of the early detectors used were unstable whilst the later detectors have a very good stability

The variance is certainly to the largest extent due to variance in the size of the non perfused zone Free P^{32} phosphate will probably not cause any essential error since this compound will be distributed in the whole body and not only in the kidney P^{32} phosphate, unlike sodium, chloride and urea is not accumulated in the medulla (Andrysek et al 1962)

The values given here agree in the main with those obtained in previous investigations (see Table 3) No direct comparison is possible, however since the division of the renal medulla into different regions is not always clearly defined in the studies referred to It shall be pointed out, however that the values obtained by the present method because of the non perfused zone rather represent an underestimation than an overestimation of the regional red cell volume Taking into account the preliminary nature of the method presented the results are not dissatisfying It may also be mentioned that the use of, for example K^{42} (3 5 MeV) as the cell label would reduce the error of the method considerably (theoretically to less than half of the error when using P^{32} as the cell label) The great advantage of the method is that it allows repeated measurements under different conditions in the kidney *in situ*

Medullary blood flow

The regional red cell flow is calculated by dividing the red cell volume times a factor 0 75 (see p 60) by the mean transit time Using $\bar{t}_m$ and t_{peak} or $t_{A/H}$ for the mean transit time determination the regional red cell flow in the outer zone was found to be 0 8 ml/min and gram tissue in outer parts of the inner zone 0 3 ml per min and gram tissue and in the papillary region 0 1 ml/min and gram tissue Assuming that the haematocrit in the blood flowing to the different regions was equal to the arterial haematocrit the regional blood flow in the outer zone was found to be 1 9 ml/min and gram tissue in the outer parts of the inner zone 0 6 ml/min and gram tissue, and in the papillary region 0 2 ml/min and gram tissue Assuming that the haematocrit in the blood flowing to the *inner zone* was half of the arterial haematocrit as was done by Ullrich et al (1961 a) the regional blood flow in the outer parts of the inner zone will be 1 2 ml per min and gram tissue and in the papillary region approximately 0 4 ml/min and gram tissue

D Summary

In this chapter a method is described for determining the regional red cell flow in the kidney The calculations were made from determinations of the regional mean transit times and regional red cell volumes In the renal cortex the mean transit time was found to be the same at all levels The mean transit time determined from an indicator dilution curve obtained from a detector position in the centre of the cortex is thus representative for the cortex as a whole The mean transit time calculated as 2 t_m refers to the mean transit time of red cells from the renal artery, through the cortical parenchyma and out into the renal vein On determining the mean transit time through the cortex itself t_m was corrected for the mean transit time in the renal artery which was estimated to be 0 3 sec The mean transit time

obtained by this correction agrees well with that calculated as $t_{A/H}$ Assuming the red cell volume to be 8 %, the cortical red cell flow was calculated to be 1 8 ml/min and gram tissue (from 2 t_m) and 2 4 ml/min and gram tissue (2 corrected t_m) thus about 2 ml/min and gram tissue By dividing this value by the arterial hae matocrit the cortical blood flow was obtained as 4 5 ml/min and gram tissue

For determination of the regional red cell flow in the renal medulla, the medulla was first divided into three zones The first comprised the outer zone the second a 5—6 mm thick imaginary layer consisting of the outer part of the inner zone and the third the papillary region The mean transit time in these regions was determined from indicator dilution curves obtained from detector positions in the middle of these regions On calculating the mean transit times through these regions from t_m (or t_{cg}), this was corrected for the time of transit of the indicator from the site of injection in the renal artery to the margin of the region This transit time was calculated on the basis of the linear rate in descending vasa recta determined from their length and that time, t_{peak} when the indicator dilution curve had reached its peak value The mean transit time was then calculated to be about 4 sec in the outer zone of the medulla 11 sec in the outer parts of the inner zone and about 30 sec in the papillary region The red cell volume was calculated to be 7 5 % of the tissue volume in the outer zone 6 3 % in the outer parts of the inner zone and 4—11 % in the papillary region About 75 % of this volume was perfused by the indicator during its first passage through the monitored volume and 25 % was perfused by internally recirculating indicator

The regional red cell flow was calculated to be 0 8 ml/min and gram tissue in the outer zone of the medulla 0 3 ml in the outer parts of the inner zone and about 0 1 ml/min and gram tissue in the papillary region Assuming that the haematocrit of the blood flowing into these regions is the same as the arterial haematocrit, the regional blood flow was calculated to be 1 9 ml/min and gram tissue in the outer zone 0 6 ml/min in the outer parts of the inner zone and 0 2 ml/min and gram tissue in the papillary region If on the other hand, the haematocrit in the blood flowing into the inner zone had a value only half that of the arterial blood the corresponding values for the regional blood flow would become twice as high as those given above

CHAPTER 7

Regional renal blood flow as related to perfusion pressure

A Material and methods

The regional blood flow in the cortex and medulla of the kidney as a function of the perfusion pressure in the renal artery, was studied in 5 dogs weighing between 17 and 26 kg and with a mean weight of 22 kg Altogether 71 indicator dilution curves from 10 detector positions were obtained

One needle detector was introduced into the middle of the cortex and one into the medulla at depths varying between 4 and 9 mm from the cortico medullary border The perfusion pressure was varied by means of a clamp arrangement placed on the aorta proximal to its junction with the renal artery The perfusion pressure was measured via a catheter placed with its tip distal to the junction with the renal artery

In the experiments the regional blood flow, in particular, was studied at pressures varying between the initial pressure (110—130 mm Hg) and 30 mm Hg In two of the experiments the blood flow was also studied at high perfusion pressures which were obtained by ligation of both carotid arteries and vagus nerves preceded by pharmacological blockade of the renal nerves by the infiltration of 5 ml Carbocain® 0 5 % (AB Nobelkrut Bofors Sweden) into the renal pedicle

B Results

The mean value of t_m in the cortex at the control pressures i e when the perfusion pressure was equal to the normal systemic pressure varied between 0 8 and 1 9 sec In the medulla the shortest transit time was 3 2 sec obtained from a recording in the outer zone and the longest 19 sec obtained from a recording in the inner zone 8—9 mm from the cortico medullary border

A typical experiment is shown in Table 5 where the results of the mean transit time determinations at a series of perfusion pressures varying between 45 and 120 mm Hg are given The indicator dilution curves obtained from the cortex and medulla at perfusion pressures of 120 and 70 mm Hg are given in Figs 26 and 27

It is evident from the table and figures that the mean transit time in the cortex expressed as t_m increased from 1 8 to 2 1 sec when the perfusion pressure was decreased from 120 to 70 mm Hg (The changes in the mean transit time in the cortex and medulla expressed as $t_{A/H}$ or t_{cg} showed essentially the same pattern) In other words the perfusion pre sure decreased by 42 % while the blood flow expressed as $1/t_m$ only decreased by 14 % This means that the blood flow in the cortex was not linearly related to the perfusion pressure but was autoregulated within this pressure range

TABLE 5 Mean transit time $\bar{t}_m$ in cortex and medulla and total renal blood flow calculated from the cortical and medullary indicator dilution curves as related to the perfusion pressure

Injection number	Perfusion pressure mm Hg	Cortex $\bar{t}_m$ sec	Medulla $\bar{t}_m$ sec	Total blood flow calculated from recordings in Cortex ml/min	Medulla ml/min
1	120	1 8	8 2	285	219
2	100	1 9	9 4	318	205
3	70	2 1	10 4	270	189
4	45	2 6	12 3	221	176
5	130	2 0	9 6	—	—

In the medulla the transit time was prolonged from 8 1 to 10 4 sec when the perfusion pressure was reduced from 120 to 70 mm Hg The medullary blood flow expressed as $1/\bar{t}_m$ was then reduced by 21 % while the perfusion pressure was reduced as before by 42 % This means that the blood flow to the medulla was autoregulated and to approximately the same extent as that to the cortex

As can be seen in the table the mean transit times in the cortex and medulla decreased when the clamp around the aorta was released and the perfusion pressure returned to the level of the systemic blood pressure The blood flow in the cortex and medulla did not then entirely regain the initial values but were 10 % and 15 % lower respectively The systemic blood pressure now rose from 120 to 130 mm Hg

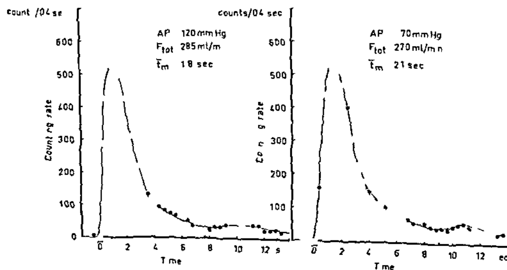
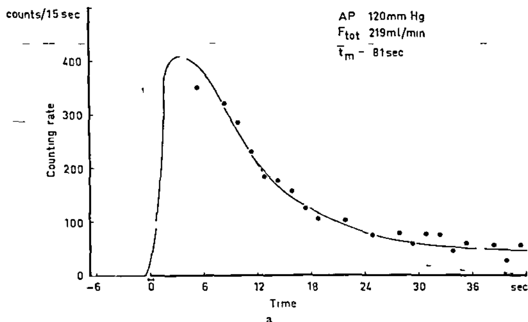
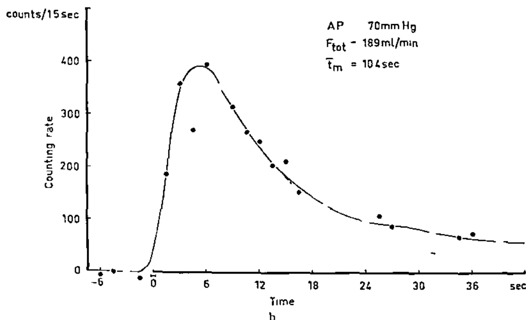


Fig 2b Cortical indicator dilution curves obtained at 120 and 70 mm Hg $\bar{t}_m$ will then increase from 1 8 to 2 1 sec The total renal blood fl...



a



b

Fig 27a and b Medullary indicator dilution curves obtained at 120 and 70 mm Hg from the same experiments as in Fig 26 $\overline{t}_m$ will then increase from 8 1 to 10 4 sec

The total renal blood flow F_{tot} as calculated from the medullary indicator dilution curves decreases from 219 to 189 ml/min Note the discrepancy between F_{tot} calculated from cortical and the corresponding medullary indicator dilution curves

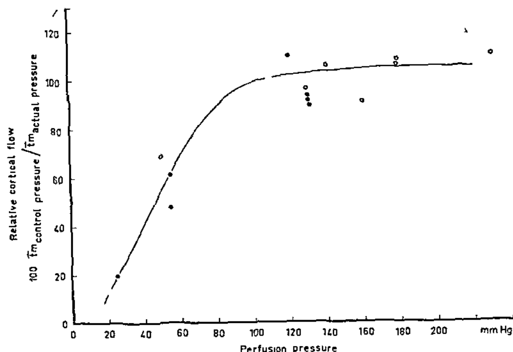


Fig 28 Relationship between regional cortical blood flow and perfusion pressure The flow values are calculated as the reciprocal of t_n and expressed in per cent of the mean blood flow value at the control pressures (110—130 mm Hg) Values obtained after ligation of the carotids are denoted by open circles The line is drawn to the best visual fit

It is clear in other words that during the experimental period the blood flow in the kidney deteriorated somewhat Assuming that the blood flow decreased successively during the clamping period the mean value of the mean transit times before and after the clamping was taken as the value for mean transit time at the control pressures The same successive reduction of the blood flow was observed in the other experiments in this investigation although to a smaller extent In one series of perfusion pressures (usually 2 series of perfusion pressures were obtained from each experiment) in one of the experiments however the discrepancy between the mean transit times at the control pressures before and after the clamping was more than 20 % Because of this large discrepancy between the values this series has been excluded from Figs 28 and 29 but is included in Fig 30

Figs 28 and 29 summarize the results from this series of experiments In these the blood flow was calculated as $1/t_m$ and expressed in per cent of the mean value of the blood flow at the control pressures as described above A prerequisite for the use of $1/t_m$ as a relative measure of the blood flow is however that the red cell volume remains unchanged under the different experimental conditions This was on the whole found to be the case at perfusion pressures varying between about 70 and 200 mm Hg When on the other hand the perfusion pressure was reduced to about

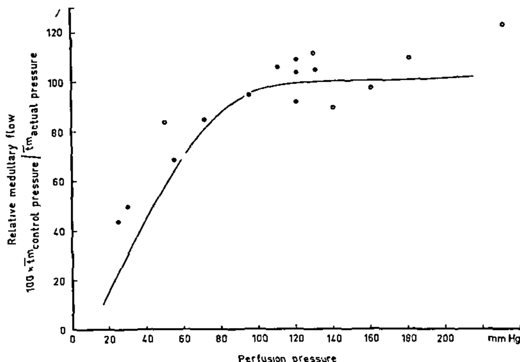


Fig 29 Relationship between regional medullary blood flow and perfusion pressure The flow values are calculated as the reciprocal of t_m and expressed in per cent of the mean blood flow at the control pressures (110—130 mm Hg) Values obtained after ligation of the carotids are denoted by open circles The curve is drawn to the best visual fit

30 mm Hg an increase of the red cell volume by 10—30 % was obtained as a rule, in the cortex while in the medulla this volume showed smaller changes In the figures no correction for these changes in red cell volume has been made It would seem evident from Figs 28 and 29 that the blood flow both in the cortex and medulla rose relatively linearly with the perfusion pressure within the pressure range between 0 mm Hg and about 70 mm Hg In the pressure range between 70 and 200 mm Hg, on the other hand the blood flow in both the cortex and medulla remained relatively constant The findings thus show that both the cortical and medullary blood flow are autoregulated In Fig 30 the relative blood flow in the cortex is compared with that in the medulla It is evident from this figure that the percentage change in the blood flow on alteration of the perfusion pressure was on the whole the same in the cortex and medulla This finding indicates that the autoregulation is approximately equally well developed in the purely cortical circulation as in the juxtamedullary circulation

In this series the total red cell flow F_{tot} in the renal artery was also determined in accordance with the equation

$$A = \frac{M_{tot}\ V_{exp}\ \overline{E}_{exp}}{F_{tot}}$$

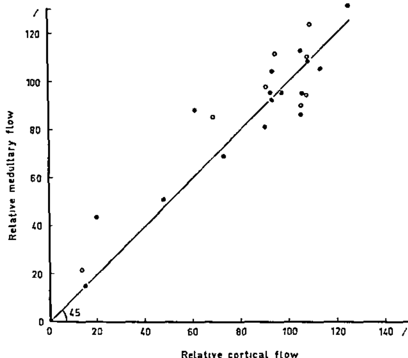


Fig 30 Comparison between relative cortical and medullary blood flows The blood flow values are calculated as the reciprocal of $\bar{t}_m$ and expressed in per cent of the mean blood flow at the control pressures The straight line is the line of identity

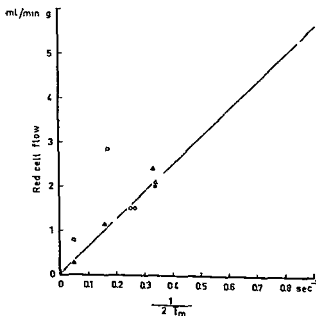


Fig 31 Relationship between regional cortical red cell flow calculated as $\frac{1}{2 \times \bar{t}_m}$ (abscissa) and that calculated from the medullary indicator dilution curves and weight of cortex (ordinate) Different symbols refer to different experiments The straight line is calculated by the method of least squares assuming the line to be $y = kx$

where A is the area of the indicator dilution curve obtained, V_{exp} the measured red cell volume, M_{tot} the amount of injected indicator, and $\bar{E}_{exp}$ the experimentally determined efficiency of the detector (cpm/μC P^{32}/ml) Note that the formula can be used both for total blood flow and total red cell flow determination (see further Chapter 4)

Γ_{tot} can then be calculated both from the cortical and the medullary indicator dilution curves In Fig 31 the Γ_{tot} values obtained from the medullary indicator dilution curves are compared with the cortical red cell flow expressed as $1/2 \times t_m$ It is evident from the comparison that the total red cell flow calculated by the equation is linearly related to the cortical red cell flow This comparison is interesting because it indicates the possibility of determining the total flow in the renal artery by means of the described method

An important requirement for a correct determination of the total renal red cell flow is, however, as mentioned previously, that complete mixing of the injected bolus in the renal artery is obtained In the present investigation this requirement was probably not completely fulfilled, which is evident, *inter alia*, by the fact that Γ_{tot} calculated from the cortical and the medullary indicator dilution curves often differed (see Table 5)

CHAPTER 8

Regional renal blood flow as related to mannitol-induced osmotic diuresis

A Material and methods

The influence of osmotic diuresis on the regional blood flow in the kidney was studied in 9 dogs weighing between 16 and 31 kg and with a mean weight of 22 kg Altogether 90 indicator dilution curves are presented from 17 detector positions in the cortex and medulla In the investigations in this series one needle detector was usually placed in the middle of the cortex and one in the outer or inner zone of the medulla The experiments were performed by giving 1—3 injections of the P^{32} labelled red cells at basal urinary flow 15 % mannitol solution[1] (Mannidex AB Pharmacia Uppsala Sweden) was then infused slowly When the urine production had become stable a further injection of the labelled cells was given The procedure was repeated after infusion of as a rule stepwise increasing quantities of mannitol

In six of the experiments changes of the red cell volume were studied by determining the background activity from the detectors before and after the mannitol infusion

B Results

It was evident from this study that mannitol induced osmotic diuresis gives relatively small and partly irregular changes of the regional circulatory conditions The results are therefore presented as a whole in Table 6 In this table the mean transit time is expressed only as 2 $\bar{t}_m$ The changes in $\bar{t}_{A/H}$ or t_{cg} exhibited essentially the same pattern From this table and from Fig 34 it can be seen that the mean transit time in the renal medulla decreased with increasing diuresis This alteration was moderate however and as a rule remained within 25 % The mean transit time in the renal cortex showed essentially the same changes with a reduction of the mean transit time with increasing diuresis The changes were smaller however than in the medulla and as a rule remained within 10 %

It is also evident from the table and from the experiment illustrated in Fig 32 that the red cell volume both in the medulla and in the cortex decreased with increasing diuresis In order to elucidate this question further the relative red cell volume was studied in one experiment (Fig 33) in which osmotic diuresis was induced by 5 grams glucose given intravenously From the curve in the lower part of the figure it can be seen that the urine production rose from an initial value of 0 2 ml/min to 1 7 ml/min within the course of 3 minutes and then decreased towards 0 1 ml/min (The initial value differs from the last value of the urine production by the fact that a small amount of mannitol had been given before the experiment)

[1] All the different solutions infused had a temperature of 38 C

TABLE 6 Mean transit time in cortex and medulla and decrease in cortical and medullary red cell volume as related to mannitol induced osmotic diuresis The mean transit times are expressed here as $2 \times \bar{t}_m$ denoting at least as regards the cortex and outer medulla the transit time through an entire region

Experiment	Arterial pressure mm Hg	Cortex $2 \times t_m$ sec	Medulla $2 \times \bar{t}_m$ sec	Urine flow left kidney ml/min	Decrease in red cell volume Cortex %	Decrease in red cell volume Medulla %	Mannitol 15 % ml
1	140	2 4	8 2	0 76			
							20
	140	2 2	7 3	0 84			
2	125	1 7	4 9*	0 83			
	125	1 7	4 1	0 83			
							50
	125	*1 5*	*4 2*	*3 30*			
	125	1 5	4 2	1 20			
	125	1 8	—	1 20			
	125	1 7	4 5	0 95			
3	140	2 6	10 1	0 24			
	140	2 8	9 4	0 24			
							10
	145	2 6	9 4	0 43			
	145	3 1	8 3	0 56			
							20
	150	3 1	8 4	0 89			
							40
	150	3 5	10 2	0 93			
							60
	150	2 7	6 8	3 00			
4	130	2 3	4 1*	0 54			
	130	2 3	4 2	0 54			
					12	8	20
	130	2 1	3 8	1 01			
					10	12	30
	130	2 2	3 9	1 60			
					6	8	30
	130	2 0	3 6	2 19			
	130	2 2	3 8	1 50			
					6	10	30
	125	2 3	3 6	1 99			
	120	2 3	3 8	1 15			
5	150	1 7	6 4*	0 22			
	—	1 8	6 7	0 22			
					11	16	20
	—	1 6	6 3	1 39			
					5	12	30

Forts Table 6

	160	1 6	5 6		1 70			
						< 5	< 5	30
	—	1 6	6 2		1 56			
	—	1 8	6 8		1 04			
6	130	—	17 9		0 43			
	130	—	17 2		0 43			
							14	80
	—	—	15 2		2 01			
							20	80
	120	—	13 3		2 20			
	120	—	17 3		1 13			
7	100	2 6	—		0 40			
	100	2 5	—		0 40			
	100	2 8	37 4		0 40			
						< 5	15	60
	100	2 8	34 6		1 95			
						< 5	13	60
	105	2 5	34 4		2 26			
						< 5	< 5	80
	100	2 7	29 8		3 74			
8	115	—	8 6	24 ?	0 95			
						8	13	40
	120	—	8 2	20 0	1 91			
						< 5	17	80
	125	—	6 6	17 6	2 28			
9	140	3 3	13 0		0 51			
						< 5	< 5	20
	135	3 2	11 4		0 64			
	130	3 0	12 1		0 64			
						10	10	40
	135	3 1	11 0		1 44			
						7	15	80
	135	3 2	11 2		3 62			

* Medulla outer zone

The urine osmolality shown in the curve in the centre of the figure, decreased from about 600 mosm/kg to about 300 mosm/kg and then rose slowly towards 1100 mosm/kg The red cell volume in the medulla shown in the upper curve in the figure decreased in the same way to about 75 % of the initial value at the same time as the urine production attained its highest value and the urine osmolality its lowest value The red cell volume then rose slowly, and within the course of half an hour reached a value of 105 % of the initial value

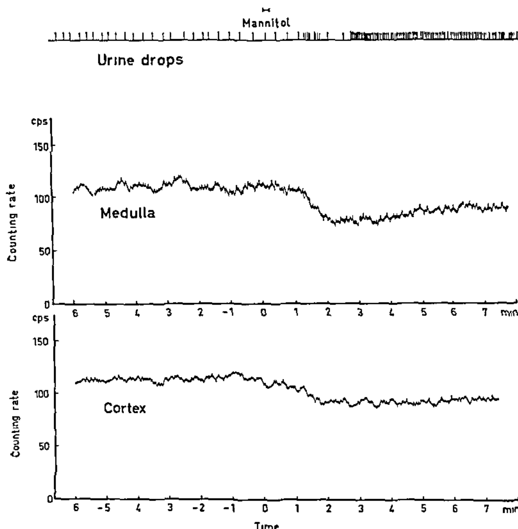


Fig 31 Background activity recorded by two needle detectors placed in cortex and medulla after the labelled cells had become equilibrated throughout the total blood volume At time zero 40 ml warm 1 mannitol is infused intravenously This will cause a decreas in both the cortical and medullary counting rates parallel to the rise in urine flow of the investigated left kidney

The curve for the relative red cell volume in the renal cortex showed the same profile in this experiment except that on maximal diuresis the red cell volume only decreased to about 90 , of the initial value

It can thus be said that in both the cortex and the medulla the mean transit time decreased with increasing diuresis and that this decrease corresponded on the whole to an approximately equally large decrease in the regional red cell volume This means thus that the blood flow expressed for example as ml/min and gram tissue should not change on induction of osmotic diuresis

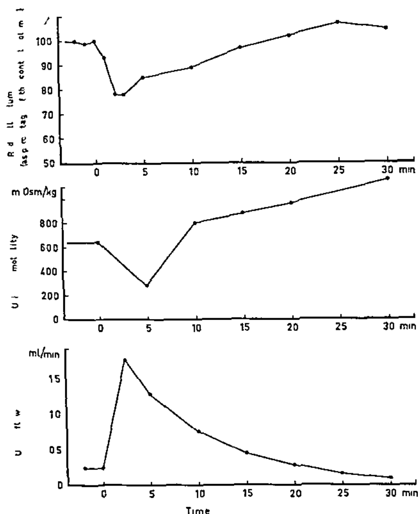


Fig 33 The influence of 5 g glucose injected intravenously on medullary red cell volume urine osmolality and urine flow It is evident from the figure that the increase in urine flow is parallel to a decrease in red cell volume and urine osmolality

The changes are small however in relation to the accuracy of this technique and no definite conclusions can be reasonably drawn therefore

One observation in this study was that the mean transit times in the cortex and medulla did not completely regain their respectively initial values when after the mannitol infusions the diuresis gradually declined This phenomenon may indicate some though moderate increase in vasoconstrictor tonus during the course of the experiment and may in that case mask an increase in the blood flow on osmotic diuresis

The glomerular filtration also decreased as a rule during the cour e of the experi ment which may also indicate the same phenomenon

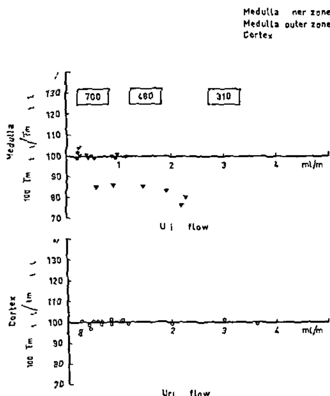


Fig 34 Cortical and medullary mean transit time of red cells calculated as $\bar{t}_m$ and expressed in per cent of the control values It is evident from the figure that the mean transit time both in the cortex and medulla will decrease with increasing urine flow The urine osmolality for urine flows between 0 and 1 ml/min averaged 700 mosm/kg for flows between 1 and 2 ml/min 480 mosm/kg and for flows exceeding 2 ml/min 310 mosm/kg

CHAPTER 9

General Discussion

A Methods for determination of regional renal blood flow

Before discussing the method described in this work a review will first be made of some of the methods used at present for analysis of the regional renal circulation The aim is to describe briefly the principles of the different methods and to discuss their validity

What is meant by a method capable of determining regional renal blood flow is actually a matter of definition, and depends upon the regions into which the kidney is divided Usually however the kidney is divided into three regions viz the cortex and outer and inner zones of the medulla A minimal criterion for measurement of regional flow is thus that the method can differentiate between these three regions In order to simplify the discussion concerning the different methods, a classification into three groups was considered justified as follows 1 Methods based on extraction of a test substance from the blood 2 methods based on the use of inert diffusible indicators and 3 methods based on the use of intravascular indicators

a Methods based on extraction of a test substance from the blood

This group includes a method based on the extraction of para aminohippuric acid (PAH) and a method based on the extraction of Rb^{86} or K^{42}

The PAH extraction method

The PAH extraction method (Reubi 1958 1961) is based on the assumption that plasma perfusing the cortical parenchyma becomes completely purified of PAH by means of active reabsorption in the proximal tubuli while plasma perfusing the renal medulla does not come into contact with PAH extracting tissue Thus it was considered that PAH clearance would determine the plasma flow in the renal cortex or rather the flow to the cortical glomeruli The difference between the total plasma flow to the kidney and the PAH clearance would then constitute the plasma flow through the juxtamedullary glomeruli and the renal medulla If for example, as in man the extraction fraction E_{PAH} is 0 9 this is interpreted as meaning that 90 % of the plasma flowing to the kidney is perfusing cortical glomeruli and 10 % juxtamedullary glomeruli This method does not permit differentiation between the outer and inner zone of the medulla

Even though this method has been used widely little investigation has been made as to its reliability No definite evidence for or against the correctness of the method has been presented Some indirect support for the method has been provided recently by Pilkington et al (1965) Schnermann and Thurau (1965) found on micropunc

ture, that the PAH concentration in vasa recta blood of golden hamsters was 4—13 times as high as in arterial blood on antidiuresis and about twice as high on osmotic diuresis These findings indicate a probably passive reabsorption of PAH from Henle s loop and collecting ducts This means that the medullary blood flow measured by this method will be underestimated and that the underestimation will probably be greater on antidiuresis than on osmotic diuresis Further, an active reabsorption in the pars recta of the proximal tubuli cannot be excluded either (Cortney et al 1965)

Results obtained by this method have been compared with those from the Rb^{86} extraction method in investigations of Harzing and coworkers (Hárzing and Bartha 1966 Harzing et al 1967) (see below) The results of the two methods were found to agree relatively well as regards osmotic diuresis in stop flow, on the other hand the medullary flow measured by the PAH extraction method increased both absolutely and in relation to the cortex while with the Rb^{86} extraction method the flow decreased both absolutely and relatively If it can be assumed that at least qualitatively the Rb^{86} extraction method with regard to the medullary blood flow is correct which has been supported by investigations with the present method (Wolgast unpublished data) and of Kramer et al (cited Thurau 1964 a), the PAH extraction method gives qualitatively erroneous values For further discussion concerning this method, reference may be made to Aukland (1962, 1964)

The Rb^{86} extraction method

The Rb^{86} extraction method (or K^{42} extraction method) for determination of regional flow was introduced by Sapirstein (1956 1958) For determination of regional renal blood flow this method has been used mainly by Hárzing and coworkers (Harzing and Pelley 1965 Harzing and Bartha 1966 Harzing et al 1966 and Harzing et al 1967) In these studies the regional flow is calculated (see Harzing and Pelley 1965) by multiplying the measured cardiac output by that fraction of the entire injected amount of Rb^{86} which has accumulated in one minute in the region in question

For a correct determination of the regional flow according to this method of calculation however two requirements must be fulfilled

1 that the concentration of the indicator is the same in the blood entering the region in question as in the central arterial blood and
2 that the concentration of the indicator in blood leaving the region in question is the same as in central venous blood

This can be shown as follows Utilizing the Fick principle the amount accumulated, M, within one minute (60 sec) after intravenous injection of a certain amount of Rb^{86} M_{tot} is obtained as

$$M = F\left[\int_{t=0}^{t=60} C_{ar}(t) - \int_{t=0}^{t=60} C_{vr}(t)\right]$$

where $C_{ar}(t)$ and $C_{vr}(t)$ are the concentrations of the indicator in the blood flowing to and from the region in question respectively and F is the flow The cardiac out

put, F_{tot}, is calculated by dividing the total amount of the isotope injected by the area of the central arterial time concentration curve up to one minute minus the area of the recirculation part of this curve, e g the central venous time concentration curve as according to

$$F_{tot} = \frac{M_{tot}}{\int_0^{60} C_{ae}(t) - \int_0^{60} C_{ve}(t)}$$

where $C_{ae}(t)$ and $C_{ve}(t)$ are the concentrations in central arterial and venous blood respectively Combining these two equations we obtain

$$\frac{M}{M_{tot}} = \frac{F}{F_{tot}} \frac{\int_0^{60} C_{ar}(t) - \int_0^{60} C_{vr}(t)}{\int_0^{60} C_{ae}(t) - \int_0^{60} C_{ve}(t)}$$

It is clearly evident that the Rb^{86} extraction method will determine the regional blood flow correctly only if the last expression is equal to one Whether this is the case with regard to the different regions in the kidney is not known

In practical experiments the total blood flow in the kidney as determined by this method has been compared with a directly measured total blood flow (venous out flow) (Harzing et al 1967) With free urinary flow the two methods were in good agreement In stop flow on the other hand the directly measured blood flow increased while the total flow calculated by the Rb^{86} extraction method showed a distinct reduction

It can thus be concluded that the two methods described here are based on assumptions which have been partly refuted and that changes observed with these methods can be ascribed both to a change in the blood flow and to a change in the extraction conditions in the studied region (Nakamura et al 1963)

b Methods based on the use of inert diffusible indicators

This group comprises methods in which the regional blood flow is calculated from the rate of transport of inert diffusible indicators such as noble gases hydrogen gas and heat[1] Detection of the indicator can be either external or internal

External monitoring

External recording of the efflux curve of a radioactive inert gas for analysis of regional renal blood flow was introduced by Thorburn et al (1963) With this method Kr^{85} (or Xe^{133}) dissolved in saline is administered as a slug injection into the renal artery The wash out curve is recorded by a gamma detector placed over the kidney According to Thorburn and coworkers this curve can be divided into four mono exponentially falling parts It was shown by an autoradiographic technique that part 1 referred to the efflux in the greater part of the cortex, part 2 to the inner part of the cortex and the outer zone of the medulla part 3 to the inner zone of the medulla and part 4 to hilar and perirenal fat

In the cortex where the arterio venous gas shunt is probably negligible (for ob-

[1] For the basic principles reference may be made to Kety 1951 Kety 1960 Ingvar and Lassen 1962

jections see Levy and Imperial 1961) it may be considered that the aim of the method is to determine the absolute blood flow In the studies of Ladefoged et al (1965) it was found that this aim was fulfilled, at least approximately

In the renal medulla the conditions are different here the gas, during the efflux phase, will be shunted over from venous vasa recta to arterial vasa recta This means that the concentration of gas in the blood passing to the region during the wash out period is not zero which is a basic prerequisite if the values obtained for the regional blood flow are to represent absolute blood flows Thus with regard to the renal medulla the method is to be regarded as a means of determining the effective or nutritive blood flow with respect to the indicator in question which also was pointed out by the authors

The vital point in the validity of this method lies probably in the accuracy with which the different efflux rates, analysed from the disappearance curve agree with the efflux rates from anatomically well defined regions For a high degree of accuracy in determination of the regional blood flow it is required that each region has a homogeneous blood flow and that the blood flows in the different regions are clearly separated from one another The investigations presented here (see Fig 13) indicate that under normal conditions the cortex has a homogeneous blood flow relatively distinctly separate from that in the medulla The outer and inner zones of the medulla do not appear to have a homogeneous blood flow however and neither do their blood flows appear to be clearly separated a finding which has also been made in studies by Aukland (1966 b) with hydrogen gas electrodes

This method has also been used for studies of the regional blood flow in circulatory disturbances such as in haemorrhagic hypotension (Carriere et al 1966 Truniger et al 1966) The accuracy in the calculation of the regional blood flow in these studies is probably less than in studies under normal conditions, since the blood flow in the cortex in particular does not seem to be homogeneous which was shown by the autoradiographic studies in these investigations Further the medullary blood flow rises in relation to the cortical blood flow which means that these regions will be less clearly distinguishable from one another It should be mentioned in this connection that the results of these studies and those of the studies of Aukland and Wolgast (1968) on the same problems and with apparently largely the same experimental conditions but with internally monitored indicators, are contradictory, at least with regard to the change in the blood flow of the renal medulla

With regard to values obtained from the inner zone of the medulla, the results are probably influenced by recirculated indicator (Ladefoged 1964)

The great advantage of this method is that it is atraumatic and permits studies even in man (e g Ladefoged and Pedersen 1967 Fritjofsson et al 1966)

Internal monitoring

This group includes various heat dilution methods (Schieve et al 1959 Sadler and Tuttle 1963, Aukland 1966a Ochwadt and Schmier 1954 Scher 1951 Perl and Hirsch 1966 Grangsjo et al 1966a Grangsjo et al 1966c Gilmore 1964) the hydro

gen clearance method (Aukland et al 1964 Aukland and Berliner 1964 Neely et al 1965) and the Kr^{85} clearance method (Aukland and Wolgast 1968) In the following discussion the hydrogen gas method according to Aukland and Berliner (1964) will be taken as a model The basic theory of this method is in essential agreement with that in external monitoring Hydrogen gas is administered by a continuous infusion — by respiration and by continuous infusion into the renal artery — until a steady state concentration has been attained in the renal parenchyma From the wash-out curve which is recorded from hydrogen sensitive platinum electrodes inserted into different parts of the kidney after discontinuation of the hydrogen administration the regional blood flow is calculated

This method has been found to give reliable values of the absolute flow in the cortex at low and normal blood flows but only exceptionally at high flows This phenomenon can probably be explained in part by the presence of a non perfused zone around the electrodes (Grangsjo et al 1966a)

In the outer zone of the renal medulla this method has been compared with the Kr^{85} clearance method and also with the method with P^{32} labelled red cells which has been described in this work (Aukland and Wolgast 1968) It was found that the blood flow calculated by the gas methods showed distinctly lower values than that calculated from the transit time for red cells and red cell volume This was to be expected since the gas methods, as mentioned previously refer to effective blood flow Calculated relative changes in the blood flow caused by haemorrhagic hypo tension retransfusion or vasoconstrictor substances[1] showed on the other hand approximately similar values With regard to the inner zone of the medulla the wash out rate of the hydrogen gas is determined to a large part by the urinary flow (Aukland and Berliner 1964)

c Methods based on the use of intravascular indicators

The aim of the methods in this group is to determine the flow of intravascular particles i e red cells and plasma All methods in the group are indicator dilution methods in which the indicator consists of labelled albumin and/or labelled red cells They may be divided according to the principles of monitoring and analysis

Compartment analysis of the indicator dilution curve obtained in the venous outflow

In this method the regional blood flow is determined as according to Ochwadt (1963) by compartment analysis of the wash out curve which is obtained in the renal venous outflow after discontinuation of a continuous infusion of donor blood containing I^{131} labelled plasma and Cr^{51} labelled red cells No blood is allowed to recirculate during the period of analysis The compartment analysis is based on the assumption that the efflux of intravascular indicators from a region is a monoexponentially falling curve

With minor modifications as regards its principles this method has been used by several authors (e g Deetjen et al 1964 Ladefoged and Pedersen 1967)

[1] (Aukland and Wolgast unpublished data)

jections see Levy and Imperial 1961) it may be considered that the aim of the method is to determine the absolute blood flow In the studies of Ladefoged et al (1965) it was found that this aim was fulfilled, at least approximately

In the renal medulla the conditions are different, here the gas during the efflux phase will be shunted over from venous vasa recta to arterial vasa recta This means that the concentration of gas in the blood passing to the region during the wash out period is not zero, which is a basic prerequisite if the values obtained for the regional blood flow are to represent absolute blood flows Thus with regard to the renal medulla the method is to be regarded as a means of determining the effective or nutritive blood flow with respect to the indicator in question which also was pointed out by the authors

The vital point in the validity of this method lies probably in the accuracy with which the different efflux rates analysed from the disappearance curve agree with the efflux rates from anatomically well defined regions For a high degree of accuracy in determination of the regional blood flow it is required that each region has a homogeneous blood flow and that the blood flows in the different regions are clearly separated from one another The investigations presented here (see Fig 13) indicate that under normal conditions the cortex has a homogeneous blood flow relatively distinctly separate from that in the medulla The outer and inner zones of the medulla do not appear to have a homogeneous blood flow, however and neither do their blood flows appear to be clearly separated a finding which has also been made in studies by Aukland (1966 b) with hydrogen gas electrodes

This method has also been used for studies of the regional blood flow in circulatory disturbances such as in haemorrhagic hypotension (Carriere et al 1966 Truniger et al 1966) The accuracy in the calculation of the regional blood flow in these studies is probably less than in studies under normal conditions since the blood flow in the cortex in particular does not seem to be homogeneous, which was shown by the autoradiographic studies in these investigations Further, the medullary blood flow rises in relation to the cortical blood flow which means that these regions will be less clearly distinguishable from one another It should be mentioned in this connection that the results of these studies and those of the studies of Aukland and Wolgast (1968) on the same problems and with apparently largely the same experimental conditions but with internally monitored indicators are contradictory, at least with regard to the change in the blood flow of the renal medulla

With regard to values obtained from the inner zone of the medulla the results are probably influenced by recirculated indicator (Ladefoged 1964)

The great advantage of this method is that it is atraumatic and permits studies even in man (e g Ladefoged and Pedersen 1967 Fritjofsson et al 1966)

Internal monitoring

This group includes various heat dilution methods (Schieve et al 1959, Sadler and Tuttle 1963, Aukland 1966a Ochwadt and Schmier 1954 Scher 1951, Perl and Hirsch 1966 Grangsjo et al 1966a Grangsjo et al 1966c Gilmore 1964) the hydro

gen clearance method (Aukland et al 1964 Aukland and Berliner 1964 Neely et al 1965) and the Kr^{85} clearance method (Aukland and Wolgast 1968) In the following discussion the hydrogen gas method according to Aukland and Berliner (1964) will be taken as a model The basic theory of this method is in essential agreement with that in external monitoring Hydrogen gas is administered by a continuous infusion — by respiration and by continuous infusion into the renal artery — until a steady state concentration has been attained in the renal parenchyma From the wash out curve which is recorded from hydrogen sensitive platinum electrodes inserted into different parts of the kidney after discontinuation of the hydrogen administration, the regional blood flow is calculated

This method has been found to give reliable values of the absolute flow in the cortex at low and normal blood flows but only exceptionally at high flows This phenomenon can probably be explained in part by the presence of a non perfused zone around the electrodes (Grangsjo et al 1966a)

In the outer zone of the renal medulla this method has been compared with the Kr^{85} clearance method and also with the method with P^{32} labelled red cells which has been described in this work (Aukland and Wolgast 1968) It was found that the blood flow calculated by the gas methods showed distinctly lower values than that calculated from the transit time for red cells and red cell volume This was to be expected since the gas methods as mentioned previously refer to effective blood flow Calculated relative changes in the blood flow caused by haemorrhagic hypo tension retransfusion or vasoconstrictor substances[1] showed on the other hand approximately similar values With regard to the inner zone of the medulla the wash out rate of the hydrogen gas is determined to a large part by the urinary flow (Aukland and Berliner 1964)

c. Methods based on the use of intravascular indicators

The aim of the methods in this group is to determine the flow of intravascular particles i e red cells and plasma All methods in the group are indicator dilution methods, in which the indicator consists of labelled albumin and/or labelled red cells They may be divided according to the principles of monitoring and analysis

Compartment analysis of the indicator dilution curve obtained in the venous outflow

In this method the regional blood flow is determined as according to Ochwadt (1963) by compartment analysis of the wash-out curve which is obtained in the renal venous outflow after discontinuation of a continuous infusion of donor blood containing I^{131} labelled plasma and Cr^{51} labelled red cells No blood is allowed to recirculate during the period of analysis The compartment analysis is based on the assumption that the efflux of intravascular indicators from a region is a monoexponentially falling curve

With minor modifications as regards its principles this method has been used by several authors (e g Deetjen et al 1964 Ladefoged and Pedersen 1967)

[1] (Aukland and Wolgast unpublished data)

The main criticism against the method seems to be that the different compartments into which the total curve is divided have not yet been identified with the efflux from anatomically well defined regions It may be pointed out, further, that the slow compartment as regards the albumin wash out can arise from extravascularly circulating albumin

Accumulation rate of albumin in the medullary tissue

Lilienfield and coworkers (Lilienfield and Maganzini 1960, Lilienfield et al 1961 Goldberg and Lilienfield 1963, Lilienfield and Pomerantz 1963) for determination of the regional medullary plasma flow, studied the rate at which I^{131} labelled albumin is accumulated in the outer and inner zones of the renal medulla

In this method a continuous infusion of I^{131} labelled albumin is given The experimental animals (dogs) are then killed at varying times between 0 1 and 3 min after the start of the infusion after which the relative amount of accumulated albumin is determined By adding together the results from such a series of dogs an accumulation curve is obtained the slope of which during the first half minute is used as a basis for the plasma flow calculation

The method is based on the assumptions that during the first half minute

1 the labelled albumin bound for the region studied has actually reached this region and
2 no labelled albumin has left the region

Whether these assumptions are reasonable or not seems to be difficult to ascertain One disadvantage of the method is further that it requires several experimental animals for *one* determination of the regional blood flow This method is nevertheless interesting since at present it would seem to be the only realistic method for determination of the regional absolute blood flow in the renal medulla apart from the method of Kramer and coworkers and the method presented in this work Thus the plasma flow in the outer zone of the medulla is found to be 180 ml/min and in the inner zone of the medulla 25—60 ml/min all calculated per 100 grams tissue (Lilienfield and Pomerantz 1963)

Internal monitoring of intravascular indicators

This group includes the present method which has been described in part previously (Wolgast 1966 Grangsjo et al 1966b) and also the well known photoelectric method developed by Kramer and coworkers (Kramer et al 1960 Thurau Deetjen and Kramer 1960 Deetjen et al 1964 Meier et al 1964 and Nizet et al 1967)

In the latter method the indicator consists of protein bound dye The dye is detected photometrically An attempt at reproducing the monitored volumes in this method has been made in Fig 35 For the sake of completeness it may be mentioned here that the photoelectric method has been complemented by a method using P^{32} labelled red cells and detection with small GM tubes inserted into the renal parenchyma (Thurau et al 1959) Further Pinter and Glaser (1967) have determined the transit time for plasma in the cortex and medulla with I^{131} labelled albumin

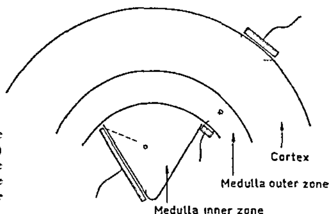


Fig 35 An attempt to visualize the monitored volumes (broken lines) in the photoelectric technique The circles shown in the figure denote the small lamps inserted into the parenchyma

and detection with semiconductor detectors As yet these two methods do not appear to have been developed in detail

The methods in this group are thus indicator dilution methods where the indicator is monitored by detectors inserted into the renal parenchyma The regional blood flow is calculated by determinations of the regional volume of red cells or plasma and the mean transit time of the indicator

In the present method the regional red cell volume is determined as the distribution volume of the indicator The validity of this method of determination depends mainly on the size of the non perfused zone around the detector In the cortex and in the region around the cortico medullary border haemorrhages are often seen around the detector which introduces an uncertainty into the determinations of the red cell volume in these regions In the renal medulla no or very small haemorrhages are seen as a rule around the detector which means that the determinations here are more reliable The values obtained are also in good agreement with those obtained by other and more acceptable methods An alternative method which could perhaps permit acceptable accuracy even in the cortex would be to use a beta emitting isotope of higher energy such as for example K^{42} as the cell label In this case the volume of the non perfused zone would probably be negligible in relation to the entire monitored volume The advantage of the reported method is that it allows repeated determinations in the living kidney

The determination of the mean transit time through the different regions in the kidney has been discussed in fairly great detail in the previous chapters and therefore only a few general points will be mentioned here It should be pointed out that the most essential requirement for a correct determination of the mean transit time through a region is that the monitored volume from a circulatory aspect can be considered representative of the region in question This means, among other things that the mean transit time in the monitored volume should be the same as in the rest of the region In the present method this problem has been solved by a mutual adaptation of the size of the monitored volume to the size, or rather the thickness of the regions into which the kidney is divided in this study

In the renal cortex this problem is fairly simple since the transit time is relatively equal at all levels in the parenchyma a phenomenon which has been described previously by Overbeck (1957) For the medullary blood flow determinations the renal medulla was first divided into the outer zone, the outer 5—6 mm thick layer of the inner zone and the papillary region The mean transit time was calculated from the indicator dilution curve obtained from a detector placed in the middle of such a region Obviously the detectors could not be placed exactly at the determined positions in the medulla The mean transit time in a region is then calculated by interpolation between values of the mean transit time obtained from detector positions at either side of the position in the middle of the region As a rule, however the mean transit time was only determined from one detector position for each injection The method would be improved essentially if the mean transit time could be determined from at least three detector positions simultaneously [1]

With regard to determination of the mean transit time, it has been shown that their validity is dependent on the circulatory conditions in the investigated region where the conditions in the renal medulla seem to be most critical The discussion will therefore be concentrated now to the determinations just in the medullary regions of the kidney

The mean transit time through a region is most simply determined as $t_{A/H}$ To judge by the distribution of the indicator in Fig 24 at the time point when the peak of the curve is recorded $t_{A/H}$ underestimates the true mean transit time through the monitored volume This is evident by the fact that the actual efficiency with which the detector records the indicator is greater than the mean efficiency It should be pointed out that this favourable distribution of the indicator occurs during a short time interval by means of the relatively high linear rate in the descending vasa recta and further that the indicator slug in the different vessels has a certain extension in time These factors will reduce the postulated error in the determination of the mean transit time as $t_{A/H}$ A further factor in this connection is that part of the area of the analysed first part of the curve may arise from internally recirculating indicator

In this study the transit time was also calculated as 2 $\bar{t}_m$ and 2 t_{cg} where t_m and t_{cg} were reduced for the transit time of the indicator up to the border of the studied region It was then shown that t_m constituted a good approximation of the time of transit from the site of injection and through half the blood volume in the monitored volume whereas t_{cg} probably overestimated this time The critical point in this method of analysis probably lies in the calculation from t_{peak} of the linear rate in the descending vasa recta It is probable that with this method the linear rate is underestimated This means that the mean transit time through the monitored volume will be underestimated if $\bar{t}_m$ is used in this method of analysis It may be noted that the results from t_m and t_{peak} calculations are generally the same as those of $\bar{t}_{A/H}$

It is very difficult to estimate the magnitude of the error in the different determina

[1] A needle detector with three separate crystals placed serially in the same needle is under development

tions of the mean transit time through a region As discussed previously, the time required for the indicator to pass to a position 8 mm from the cortico medullary border would seem to be overestimated by less than half a second This should mean that the mean transit time calculated from t_m and t_{peak} will be underestimated by about 10 % or less a value which thus also holds for the $t_{A/H}$ determination If it is taken into account that with this method the calculated red cell volumes rather represent an underestimation than an overestimation of the true red cell volume the regional blood flow would seem to be relatively correctly determined

The regional blood flow was calculated by dividing the determined red cell flow by the haematocrit value[1] in blood flowing to (or from) the region in question With regard to the blood flowing to the cortex (i e in the renal artery) this haematocrit value is equal to that in central arterial blood In blood flowing to the regions in the medulla it has been postulated that the haematocrit value should be low due to a cell separation process in the interlobular arteries leaving a cell poor fraction of blood to perfuse the juxtamedullary glomeruli while the superficial glomeruli are perfused with a cell rich fraction (Pappenheimer and Kinter 1955) The magnitude of this cell separation seems to be small however Ulfendahl (1962 b) found the haematocrit value in blood drawn from veins draining the superficial layers of the cortical parenchyma to be only about 10 % greater than in central arterial blood This difference has also been explained by the possibility that the fluid reabsorbed from the fluid filtered in the superficial glomeruli is partly delivered to peritubular capillaries draining into the deep venous system (Nissen 1965 1966)

By direct puncture of vasa recta in golden hamsters it was found that the haematocrit value (corrected for the shrinkage of the red cells in the hyperosmotic environment in the medulla) was about half of that in central arterial blood (Ullrich et al 1961c) Due to the technical difficulties involved in the micropuncture technique, the values obtained have to be interpreted with caution No definite decision as to which value of the haematocrit shall be used in the calculation of blood flow in the renal medulla has therefore been made especially as it seems possible to determine both the red cell flow and the plasma flow by the indicator dilution technique described in this work

For comparison, a few points regarding the *photoelectric technique* will also be discussed here briefly With this technique the blood volume has been calculated from determinations of the regional red cell volume and published data on the intravascular haematocrit The red cell volume has been calculated from the regional haemoglobin concentration measured photometrically (Kramer et al 1960) or by chemical analysis (Deetjen et al 1964) With regard to the monitored volumes in the photoelectric technique an attempt has been made to show these in Fig 35 It can be seen that the cortical detector monitors the outer layer of this region As discussed pre

[1] The value referred to here is that haematocrit value that gives a ratio of the red cell flow to that of whole blood determined for example by centrifugation of blood drawn from a vessel via a catheter cannula or similar means and not the haematocrit value that gives a ratio between volumes determined from for example the volume of distribution of labelled red cells and volume of distribution of labelled albumin

viously however the mean transit time through this part of the cortex is probably more or less the same as that through the cortex in its entirety This is also supported by a study of Pedersen et al (1965) where it was shown that the mean transit time measured in the renal vein and the mean transit time through the cortex expressed as 2 t_{cg} were in good agreement

As regards the outer zone only the inner (half?) layer of this region is monitored The monitored volume can hardly be regarded as representative of the outer zone as a whole In the inner zone an equally large proportion of the outer parts as of the papillary region is monitored In actual fact the papillary region constitutes the clearly smaller portion of the inner zone volume Consequently the mean transit time in the inner zone will be overestimated

The mean transit time has been calculated as that time which in the present study has been denoted by t_{cg} Certain correction for the transit time up to the monitored volume has been made

The blood flow values obtained by the method as summarized by Ullrich et al (1961a) amount to 5 35 2 33 and 0 38 ml/min and gram tissue for cortical, outer medullary and inner medullary parenchyma respectively

B Biological results

a Regional renal blood flow

It is evident from these investigations that the blood flow in the cortex is high and amounts to about 4 5 ml/min and gram tissue It seems further to be equal at all levels in this parenchyma

In the medulla the blood flow decreases with increasing distance from the cortico-medullary border The blood flow in the region nearest to the cortico medullary border is probably the same as in the cortex whereas the blood flow in the papillary region amounts to only 5—10 % of the cortical blood flow

Assuming that the outer zone of the medulla comprises 20 % of the total renal weight (von Mollendorff and Schroder 1930) it is calculated that about 90 % of the total amount of blood which perfuses the kidney is distributed to cortical glomeruli and peritubular capillaries and 10 % to juxtamedullary glomeruli and the medullary vascular system The reason for the relatively low medullary blood flow is probably that the vasa recta which in the dog are up to 20 mm long create a high resistance to the blood flow A contributory reason can also be a successively increasing viscosity of the blood down towards the tip of the papilla resulting from shunting of water between the vasa recta limbs

As regards the cortex and outer medulla the values obtained are in agreement with those obtained by Lilienfield et al (see Lilienfield and Pomerantz 1963) and by Kramer et al (see Ullrich et al 1961 a) In the inner zone Kramer et al obtained a value of 0 38 ml/min and gram tissue whilst the value obtained with the present method, calculated in a similar manner (assuming the haematocrit in vasa recta blood to be half of that in central arterial blood) was 1 2 ml/min and gram tissue for

the greater part of the inner zone thus 3—4 times greater Kramer's value agrees better with the value of 0 4 ml/min and gram tissue obtained with the present method for the papillary region Lilienfield obtained a value of 0 25—0 60 ml/min and gram tissue for the inner zone

The linear velocity for red cells was calculated to be 2 5 mm/sec in descending vasa recta bound for the middle of the outer zone and 1 5 mm/sec in descending vasa recta bound for the papillary region

Meier et al (1964) found a mean linear velocity of 0 65 mm/sec for plasma particles determined from the indicator dilution curve obtained from a small photocell attached to the papilla, and calculated by dividing the length of vasa recta bound for the papillary region by that time which in the present work has been designated as t_{cg} At the end of time t_{cg} however more than half of the indicator dilution curve had been recorded which according to the theories for the generation of the indicator dilution curve in the case of internal monitoring which have been discussed in this work (see chapter 4) should mean that the blood volume in the descending vasa recta is greater than the sum of the volumes in the capillary system and ascending vasa recta within the monitored volume an assumption which does not seem probable In the golden hamster Steinhausen (1964) calculated the linear velocity for plasma particles in descending vasa recta to be 0 5 mm/sec

b Regional renal blood flow as related to perfusion pressure

The present investigations of the cortical and medullary blood flow as a function of the perfusion pressure indicate that the blood flow in these two regions is relatively constant when the perfusion pressure varies between 70 and about 200 mm Hg i e both the cortical and medullary blood flows are autoregulated within this pressure range This interpretation is based on the finding that both the mean transit time and the red cell volume were approximately constant within this pressure range It may be pointed out in this connection that the values obtained refer to the regional blood flow expressed as ml/min and gram (or ml) tissue or an equivalent arbitrary measure The total flow to a region is obtained by multiplying this value by the weight or volume of the region in question which may vary under the different experimental conditions This means that the total blood flow to a region may have changed even if the measured blood flow per gram tissue remains constant Within the range of autoregulation however this does not seem to be the case since according to Swann (1964) the distended kidney volume remains fairly constant within this pressure range On reduction of the perfusion pressure towards 30 mm Hg a moderate increase (10—30 %) of the regional red cell volume was noted this being most pronounced in the cortex This change is compensated by a decrease in the distended kidney volume of approximately the same order of size (Swann 1964)

Autoregulation of the total renal blood flow is a phenomenon which has long been well known (Johnson 1964) and which has been established both in intact kidneys *in situ* and in fully isolated kidneys perfused with whole blood or plasma (e g Thurau

and Kramer 1959 a 1959 b Ochwadt 1956, Waugh 1958 1964, Waugh and Shanks 1960, Hinshaw 1964, Thurau and Henne 1964)

The relationship between the medullary blood flow and the perfusion pressure in the renal artery has been studied very little on the other hand Thurau, Deetjen and Kramer (1960) using a photoelectric technique found that the medullary blood flow (primarily corresponding to the blood flow in the inner medulla) was linearly related to the perfusion pressure while the cortical blood flow exhibited good autoregulation Replotting of the data given in this study, in the manner outlined in Figs 28 and 29 showed however that most of the experiments were consistent with an autoregulation of the medullary blood flow, although much less pronounced than in the cortex Aukland (1966 b), using a hydrogen wash out technique, found, however that the medullary blood flow (outer zone) was autoregulated and to the same extent as the total renal blood flow

Any explanation of the discrepancy between the results obtained in these investigations is highly speculative It is possible that the trauma caused by insertion of the photoelectric device may disturb the medullary autoregulation mechanism Further it is known that in deep barbiturate anaesthesia the autoregulatory capacity deteriorates (Waugh 1964) It is possible that the medullary circulation may be more sensitive to this effect

The present investigation permits no important conclusions to be drawn with regard to the background mechanism of the autoregulation phenomenon It possibly contradicts the theory advocated by Thurau (1963 1964a 1964b) and Schnermann et al (1966) that the sodium concentration at the macula densa segment of the nephron determines the precapillary resistance with renin angiotension as the transmittor system According to this theory the medullary blood flow is not autoregulated, since the renin concentration per juxtamedullary apparatus is very low in the inner part of the cortex where the juxtamedullary glomeruli are localized (Bing and Wiberg 1958 Peart 1959 Brown et al 1963)

c Regional renal blood flow as related to mannitol induced osmotic diuresis

The investigations in this series of experiments show that the mean transit time in both cortex and medulla decreases with increasing diuresis parallel with a reduction of the measured red cell volume The changes are most pronounced in the medulla of the kidney These results are on the whole in agreement with previous findings Thus Thurau Deetjen and Kramer (1960) using the photoelectric technique found that the transit time in the medulla decreased by an average of 25 % in glucose induced osmotic diuresis The transit time in the cortex remained unchanged

Goldberg and Lilienfield (1963) found in contrast no significant differences in the inflow of labelled albumin on change-over from antidiuresis to mannitol induced osmotic diuresis The size of the exchangeable albumin pool decreased on the other hand by about 20 % which is comparable with the decrease in the regional red cell volume found in the present investigation

The results of these three investigations can be interpreted as follows In antidiuresis the descending vasa recta blood gains successively a high solute concentration during its passage towards the papilla by passive inward transport of solute from the hyperosmotic interstitium and by an outward passive transport of water to the interstitium The blood flowing in ascending vasa recta will loose successively its high solute concentration by the outward transport of solute and inward transport of water derived from the descending vasa recta and from the medullary tubuli and collecting ducts The inflow of fluid to ascending vasa recta from tubuli and collecting ducts amounts to about 20 % of the total medullary blood flow, as calculated by Ullrich et al (1961 a)

By means of the outward transport of water from descending vasa recta the linear rate decreases successively, parallel with an increase in the concentration of non permeable intravascular particles such as, for example red cells In ascending vasa recta the linear rate increases and the concentration of impermeable particles decreases

Experimental evidence of a water shunt between the limbs of vasa recta as outlined above has been obtained from micropuncture studies, in which the protein concentration of vasa recta blood has been found to increase from the base to the tip of the papilla (Thurau Sugiura and Lilienfield 1960 Wilde et al 1963) Ullrich et al (1961b) found in contrast that the protein concentration in vasa recta blood was slightly higher than that in systemic blood and unaffected by the osmolarity of the medullary interstitium Wilde and Vorburger (1967), on measuring the concentration of Evans blue in the medullary vessels microspectrophotometrically on tissue slices from golden hamsters found a successive increase of up to four times in the albumin concentration with increasing distance from the cortico medullary border

In osmotic diuresis the osmolarity in the medulla is decreased (Malvin and Wilde 1959) Under this condition the amount of water shunted between the limbs of vasa recta will be reduced If, now the volume in vasa recta remains unchanged the reduced amount of water shunted across the limbs of vasa recta together with a possible increase in the amount of fluid reabsorbed from tubuli and collecting ducts (Berliner and Bennett 1967) will cause an increased linear rate of the impermeable intravascular particles parallel with an equal reduction of the concentration of the particles i e assuming that the flow of the inflowing blood remains constant at a change over from antidiuresis to osmotic diuresis This is in fact what has been found in the present experiments The transit time for red cells decreases with increasing osmotic diuresis at the same time as the red cell volume decreases The conclusion drawn from the present investigations as also from those of Thurau Deetjen and Kramer and of Goldberg and Lilienfield, is thus that the inflow of blood does not change during osmotic diuresis as compared to during antidiuresis However taking into account in the calculation the increase in kidney weight of about 20 % found in the rat on mannitol induced osmotic diuresis (*Brunner et al 1958*) and of about 10 % found in the dog on urea diuresis (Swann and Ormsby 1959) there may be a slight increase in the total renal blood flow and perhaps also in the

medullary blood flow No definite conclusions can be drawn however, since the changes in mean transit time are small in relation to the accuracy of the technique used

It should be pointed out in this connection that osmotic diuresis usually leads to an increased total renal blood flow (Braun and Lilienfield 1963 Thurau 1964 a, Thurau 1964 b) This phenomenon has been attributed to the increased interstitial pressure found in osmotic diuresis which in turn, as according to the myogenic hypothesis for renal autoregulation (see Folkow 1964), causes a decreased vascular resistance

General summary

General principle

In this work a new indicator dilution method for the determination of regional renal blood flow is presented P^{32} labelled red cells were used as the indicator the radioactivity being monitored with either beta sensitive end window semiconductor detectors (for surface monitoring) or beta sensitive needle shaped semiconductor detectors (for internal monitoring)

The experiments were carried out by injection of the labelled cells suspended in their own plasma as a slug into the renal artery From the indicator dilution curves recorded by the detectors the mean transit time for the indicator was calculated The regional red cell volume was determined after the labelled cells had been equilibrated in the total blood volume of the animal and was calculated as the arterial haema tocrit multiplied by the ratio between the counting rates recorded by the detector in the kidney and by the same detector immersed in blood withdrawn on the same occasion The regional red cell flow was obtained by dividing the regional red cell volume by the mean transit time for the labelled cells through the region under study The regional blood flow was calculated by dividing the red cell flow by the haematocrit of inflowing blood

Recording equipment

The pulses from the detectors were amplified by charge sensitive preamplifiers and linear amplifiers and recorded on scalers with automatic print out

The detecting system was found to have a very good stability and an acceptable linearity The counting rate recorded from the needle detectors mostly used when immersed in a P^{32} solution was found to be about 5000 cpm/μCP^{32}/ml

The monitored volume using P^{32} as the indicator label can be considered to correspond to an oblate spheroid with a major axis of 7 5 mm perpendicular to the long axis of the detector and a minor axis of 5 5 mm About 85 % of the total number of counts will arise from the monitored volume defined as above

Generation of the indicator dilution curve

From theoretical considerations it was found that the area of the indicator dilution curve obtained by internal and external monitoring was proportional to the amount of radioactivity injected into the renal artery and to the volume of distribution of the indicator, and inversely proportional to the flow in the renal artery The fraction of counts arising from a single capillary (pathway) is dependent on the volume of the capillary and on its location in the monitored volume and is *not proportional to the fraction of radioactivity perfusing the capillary in question*

Theoretical aspects on the determination of mean transit time

The mean transit time has been calculated as $t_{A/H}$ t_m and t_{cg} $t_{A/H}$ is calculated as the time obtained when the area of the indicator dilution curve is divided by the height of the curve, $\bar{t}_m$ is the time from the time of injection to the time when half the area of the indicator dilution curve has been recorded, and t_{cg} is the time from the time of injection to the time at which the centre of gravity of the curve occurs $t_{A/H}$ is intended to represent the mean transit time through the monitored volume and t_m and t_{cg} the mean transit time from the site of injection and through half the blood volume in the monitored volume

From theoretical studies as well as investigations on a hypothetical model system it was found that none of the methods used in general will correctly determine the true mean transit time $\bar{t}_m$ and to some extent $t_{A/H}$ constitute however rather good approximations whilst t_{cg} probably overestimates this time somewhat

Experiments on the circulatory pattern in the dog kidney

In the cortex the indicator dilution curves obtained from detector positions at different distances from the cortico medullary border were found to be of different shape The calculated mean transit times were however equal at all levels in this parenchyma It was concluded that the mean transit time determined from the curve recorded by a detector placed in the middle of the cortical parenchyma was representative of the mean transit time in the cortex as a whole The transit time from the renal artery to the renal vein calculated as 2 t_m was found to be 2 6 sec The red cell flow assuming a red cell volume of 8 % was then obtained as 1 8 ml/min per gram tissue The transit time through the cortical parenchyma itself calculated as 2 t_m where t_m was reduced for the transit time in the arteries below the cortex (estimated at 0 3 sec) was found to be 2 0 sec When calculated as $t_{A/H}$ the value obtained was 2 1 sec Assuming the same red cell volume (8 %) the red cell flows were obtained as 2 4 and 2 3 ml/min and gram tissue using t_m and $t_{A/H}$ respectively Thus the cortical red cell flow was found to be about 2 ml/min and gram tissue corresponding to a blood flow of about 4 5 ml/min and gram tissue

In the medulla the transit time expressed for example as t_m, increased from 2 5 sec in the middle of the 4—6 mm thick outer zone to about half a minute near the tip of the papilla thus showing a completely different pattern from that in the cortical parenchyma When calculating the mean transit time in the different regions of the medulla this was first divided into the outer zone the outer 5—6 mm of the inner zone and papillary region A detector placed in the middle of such a region was considered to monitor a volume segment representative of the region in question When using t_m or t_{cg} correction for the transit time of the labelled cells from the site of injection to the border of the region studied has to be made this correction being determined from the linear velocity for red cells in descending vasa recta This linear velocity was calculated from the lengths of vasa recta and that time point when the peak of the curve was recorded (t_{peak}) the latter time was found to correspond to

the time when the indicator bound for the capillaries located around the centre of the detector was just about to pass into these capillaries The mean transit time in the three regions calculated from t_m and t_{peak} was then calculated as 3 9 sec, 11 sec and 0 5 min These values fit well with those calculated as $t_A/_H$ whilst those from t_{cg} and t_{peak} were somewhat greater

The red cell volume was found to be 7 5 % in the outer zone and about 6 % in the inner zone up to 12 mm from the cortico medullary border, which comprised 80—90 % of the inner zone About 75 % of the volume was perfused by the indicator during its first transit through the region, and 25 % by internally recirculation indicator

The red cell flows in the outer zone the outer part of the inner zone and the papillary region were then calculated as 0 8 0 3 and about 0 1 ml/min and gram tissue, and the corresponding blood flows assuming the haematocrit in the inflowing blood to be equal to the arterial haematocrit 1 9 0 6 and 0 2 ml/min and gram tissue The values given represent mean values for the regions in question The linear velocity was found (from t_{peak}) to be 2 5 mm/sec in the descending vasa recta bound for the outer zone and about 1 5 mm/sec in those bound for the papillary region

It can be concluded that the determination of regional renal blood flow is very complex and involves several analytical stages

Regional renal flow as related to perfusion pressure and mannitol induced osmotic diuresis

The method was also used for studies of the influence of perfusion pressure and mannitol induced osmotic diuresis on regional renal blood flow

It was found that the blood flow in the cortex and medulla expressed as the reciprocal of t_m (or $t_A/_H$ or t_{cg}) was essentially unchanged when the perfusion pressure was varied between 70 and 200 mm Hg Below 70 mm the blood flow was relatively linearly related to the perfusion pressure i e both the cortical and medullary blood flow was autoregulated The red cell volume was essentially unchanged within the autoregulation pressure range (about 80—200 mm Hg) whilst at lower pressures it increased No correction for this change was performed

In mannitol induced osmotic diuresis the transit time both in the cortex and medulla was found to decrease moderately (10 % and 25 % for cortex and medulla respectively) with increasing urine flow This was followed by a decrease in the regional red cell volume of the same order of magnitude This was interpreted as meaning that the regional blood flow (ml/min and gram tissue) remained essentially unchanged Taking into account an increase in the total volume of the kidney during osmotic diuresis there may be a slight increase in the blood flow to the regions The changes were small, however in relation to the accuracy of the method and no definite conclusions can therefore be drawn.

General discussion

In this section some of the methods used at present for the determination of regional renal blood flow are discussed These can be divided into three main groups

1 methods based on extraction of a test substance from the blood
2 methods based on the use of inert diffusible indicators and
3 methods based on the use of intravascular indicators

The principles of the different methods are reviewed briefly From examination of the methods it is concluded that those in the first group have the advantage of being technically simple but that as yet their validity has not been verified acceptably The inert gas methods should be regarded as methods for the study of effective or nutritive flow at least where the medulla is concerned The methods in which intravascular indicators are used are all (including the present method) technically complicated and involve great analytical difficulties

The biological results obtained in this work are discussed and are compared with those reported by other investigators

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THE OCCURRENCE OF BIOGENIC MONOAMINES IN THE MAMMALIAN ENDOCRINE PANCREAS

BY

LENNART CEGRELL

LUND 1968

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CONTENTS

INTRODUCTION

Almost one hundred years ago Paul Langerhans described the Zellhaufen in pancreas which were later to be called the islets of Langerhans A vast amount of work has since then been devoted to these cell groups and it is now well established that they are built up of different cell types, which produce at least two hormones insulin from the B cells and glucagon from the A cells The role of the islet cells in the pathogenesis of diabetes is still poorly understood as are the mechanisms regulating the synthesis storage and release of the hormones The influence of the autonomic nervous system on the endocrine cells has long been discussed It is well known that the isleTs in many mammals receive an abundancy of autonomic fibres both adrenergic and cholinergic There is evidence for the view that these nerves are intimately related to islet cells but the opinions regarding their function and distribution in relation to the different cell types are contradictory (i a Bargmann 1939 Sergeyeva 1940 Heinzen 1960, Watari 1968 Esterhuizen et al 1968) During recent years a growing interest has been focused on the effect of catecholamines on the regulation of the insulin release from the B cells *In vitro* studies by Coore and Randle (1964) have demonstrated that the insulin release induced by glucose is inhibited by A* In man an inhibition of the insulin release can be evoked by infusion of A (Porte et al 1966 Karam et al 1966) or NA (Porte and Williams 1966) Similar inhibition by A has also been obtained in the rhesus monkey (Kris et al 1966) and in the dog (Campbell and Rastogi 1966) Wong et al (1967) demonstrated a marked inhibition of insulin release *in vitro* with DA NA and A Adrenaline infusion into the arterial supply to the pancreas has been shown to provoke a damage of the B cells in dog and rat whereas pretreatment of the animals with dihydroergotamine—but not with propranol—protects the cells from this effect (Loubatieres 1965) A new approach to the interpretation of the influence of monoamines in the endocrine pancreas was made in recent studies (Falck and Hellman 1963 1964 Cegrell et al 1964) which demonstrated by means of the histochemical fluorescence method of Falck and Hillarp that monoamines occur *within* endocrine pancreatic cells in many species

The aim of the present investigation was to obtain basic information necessary for a continued research into functional problems i e to determine the type of endocrine monoamines and to study their occurrence as well as the distribution of adrenergic islet nerves in different mammalian species and during different stages of development

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* Abbreviations used A = adrenaline NA = noradrenaline DA = dopamine 5-HTP = 5 hydroxytryptophane and 5 HT = 5 hydroxytryptamine

Histochemical and histological methods The fluorescence method of Falck and Hillarp (Falck 1962 Falck et al 1962 Corrodi and Hillarp 1963 1964) makes it possible to demonstrate at the cellular level certain biogenic monoamines and some closely related substances The method is based on the principle that in freeze dried tissues exposed to formaldehyde under special conditions, certain catechol and indole derivatives react with the formaldehyde and form intensely fluorescent products which can easily be localized under the fluorescence micro scope The condensation reaction takes place at the cellular storage sites of the amines without detectable displacement The method is highly sentitive and spe cific and the chemical background is well understood (see Corrodi and Jonsson 1967) Briefly, the procedure is Small tissue specimens are taken from the ani mals as soon as possible *post mortem* quenched in liquid propane cooled by li quid nitrogen freeze dried and treated in formaldehyde for one hour to de monstrate 5 HT and primary catecholamines such as DA and NA and for three hours to demonstrate secondary catecholamines such as A The dried specimens are embedded in paraffin and thereafter sectioned for fluorescence microscopy (for further details see Falck and Owman 1965) To check the occurrence of autofluorescence some tissue specimens were treated as described above but the formaldehyde exposure was omitted Fluorescence that could be attributed to the *action of formaldehyde is referred to in this work as specific fluorescence*

The spectral characteristics of the monoamine fluorescence have been ana lyzed by Caspersson et al (1966) The catecholamine fluorophores show excita tion/emission peaks at 410/470 mμ and 5 HT at 390/520 mμ In the present studies such microspectrographic analyses have been performed on a modified Leitz microspectrograph To differentiate between DA and NA the sections were treated in HCl vapour DA then gives an excitation peak at 370 mμ which persists after prolonged treatment whereas such a peak could occur for only a short time when the fluorophores originate from NA (Bjorklund et al 1968) The technical procedure has been somewhat modified the sections were fixed to the slides with a very thin layer of albumin glycerine and then lighly depa raffinized in xylene In these deparaffinized sections some seconds of HCl treat ment (as described by Bjorklund et al 1968) was enough to obtain a shift in the excitation spectra of DA By mounting the sections in xylene to facilitate the recovery of the cover glass the excitation spectra of one and the same cell could be obtained before as well as after one or more treatments with HCl The ex

posure to HCl must be shorter than three minutes in order to avoid too much auto fluorescence When the HCl treated sections were exposed to NH_3 vapour for one minute the peak of the excitation spectra of DA returned to the original

In studies aimed at identifying the monoamine-containing cells in the pancreas the sections were fixed to the glass slides with a small amount of albumin glycerine and mounted in xylene liquid paraffin or immersion oil to facilitate the recovery of the cover glass The A_1 cells were demonstrated by silver impregnation (Hellman and Hellerstrom 1960), the B cells were stained with aldehyde fuchsin as modified by Jennings (1965) Both procedures could be performed on one and the same section since the initial step in the aldehyde fuchsin staining led to a complete removal of the silver impregnation For comparison microphotographs were taken from one and the same islet of one and the same section the first of the fluorescent cells the second of the silver positive A_1 cells and the third of the aldehyde fuchsin positive B cells (silver impregnation was omitted in some experiments)

Chemical methods Pancreatic tissues used for spectrofluorimetric determinations of catecholamines were homogenized in perchloric acid the extracts passed through a cation exchange column and the catecholamines eluated by hydrochloric acid and estimated spectrofluorimetrically This procedure was performed according to Bertler et al (1958b) as modified by Haggendal (1963) 5 HT was determined as described by Bertler (1961)

For further establishing the identity of the catechol derivatives chromatographic analyses were performed in various systems according to Bertler et al (1958a)

MONOAMINES IN THE PIG PANCREAS WITH SPECIAL REFERENCE TO THE ENDOCRINE PART

by

*Lennart Cegrell, Bengt Falck and Evald Rosengren**

There is some evidence that biogenic monoamines play a functional role in several hormone producing cell systems (cf Falck and Owman 1968) In the islets of Langerhans a storage of monoamines has been demonstrated in many species (Falck and Hellman 1963, Cegrell et al 1964) Recent investigations of these endocrine monoamine mechanisms have disclosed some remarkable features (Cegrell et al 1967, Cegrell 1967 a b 1968 b) which have necessitated an expansion of the investigation to include comparative studies of more mammalian species in order to improve the possibilities of interpreting their functional significance Thus there are great species differences with regard to the identity and concentration of the monoamines found and they are not even demonstrable in all mammalian species hitherto investigated Moreover in those species where monoamines occur in the endocrine pancreas pronounced inter individual variations in their concentration are often recorded Further at least in the newborn guinea pig rabbit and mouse there are considerable differences in the monoamine concentration between the albinos and the pigmented animals It is also conceivable that one and the same islet cell can store two monoamines, 5 HT and a catecholamine In the guinea pig this catecholamine is presumably DA

This paper presents data regarding the identity and cellular localization of monoamines in the pancreas of the pig which has been choosen as a suitable experimental animal for a current series of investigations into the functional relationship between the monoamines and hormone production in the endocrine pancreas

MATERIALS AND METHODS

The material comprises three groups one consisting of 16 animals 8—10 weeks old killed in the laboratory by bleeding in pentobarbital sodium anesthesia The second group includes pancreatic glands obtained from 10 fattened

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pigs at the slaughter house The age of the animals in this group was approximately 5 months In addition, the pancreas from four newborn pigs (less than 24 hrs old) were included

Chemical methods To identify catechol derivatives parts of the pancreas from two fattened pigs were chromatographed according to Bertler et al (1958 a) in phenol N HCl (85 15)

Catecholamines were determined according to Bertler et al (1958 b), as modified by Haggendal (1963) In the newborn animals the method of Anton and Sayre (1964) for the determination of dopa and DA was used 5 HT was determined as described by Bertler (1961) The material includes the glands from all the 16 young animals part of 8 glands from the fattened pigs and the glands from two newborn animals (see Table 1) Five of the glands from the young animals were divided into two portions approximately equal in size the duodenal and the lienal part and their contents of catecholamines were estimated (Table 2)

Histochemical methods From all the animals small tissue pieces were taken from different parts of the gland and processed for fluorescence microscopy according to the method of Falck and Hillarp (see General Methods)

RESULTS

In the tissue specimens processed without formaldehyde treatment there occurred no autofluorescence of such a degree as to interfere with the microfluorimetric analyses or the observation on the specific formaldehyde induced fluorescence

Varicose fibres displaying a specific green fluorescence were found around vessels and scattered among the exocrine acini In amounts and distribution they were similar to those recently demonstrated in the exocrine parenchyma of other species (Alm et al 1967) Few adrenergic nerves were found within the islets of Langerhans Infrequently small ganglia were found scattered in the exocrine tissue and consisting of non fluorescent cell bodies surrounded by varicose adrenergic nerves Occasionally though rarely few small intensely green fluorescent cells were found in such ganglia

Enterochromaffin cells in the epithelial lining of the pancreatic ducts were sparse and were not found in every tissue specimen They showed the typical yellow fluorescence and flask shaped appearance of 5 HT containing 'enterochromaffin cells in other locations and species (cf Falck and Owman 1968)

Irregularly distributed in the exocrine tissue isolated cells occurred morphologically resembling those found in the islets Some of them showed a weak to moderate fluorescence in the green to yellow range whereas others emitted a strong green or yellow fluorescence In the slaughter house material they seemed to be rare whereas the newborn pancreas possessed them in abundance The 8—10 week old animals seemed to represent an intermediate stage in this respect

In the islets of Langerhans most of the cells displayed a specific fluorescence, usually only weak to moderate and in the green to yellow range In every animal examined, cells with an intense green fluorescence were also found in the islets Their number varied widely in the different islets some islets contained none at all In the islet cells, as well as in the cells scattered in the exocrine parenchyma the fluorescence was sometimes seen to be confined to cytoplasmic granules (cf Cegrell and Falck 1968) Some of the islets cells appeared dark in the fluorescence microscope (Fig 3)

Subsequent staining with aldehyde fuchsin revealed that probably all B cells displayed a specific fluorescence and also that many aldehyde fuchsin negative cells were fluorescent (Fig 3)

Even in the 5 month old animals a few aldehyde fuchsin positive cells were found among exocrine cells The silver positive A_1 cells were found at the periphery of the islets as described by Alm and Hellman (1964) So far, no specific fluorescence has been observed in these cells

In the microspectrograph, the islet cells and the extra insular scattered cells displaying a strong green fluorescence showed activation and emission spectra typical for catecholamines and coinciding with those obtained from the adrenergic nerves in the same sections However, after exposure of the sections to HCl vapour according to Bjorklund et al (1968) there appeared a peak of the activation spectra of these cells at 375 mμ (see Fig 1) implying the presence of DA whereas this peak did not appear in the adrenergic nerves whose fluorescence gradually disappeared as occurs in noradrenergic nerves elsewhere in the body The intensely yellow fluorescent cells which appeared scattered in the parenchyma showed fluorescence characteristics identical to those of the enterochromaffin cells in the pancreatic ducts Of the cells which exhibited a weak to moderate fluorescence in the green to yellow range only those having the stronger intensity could be analyzed in the microspectrograph These cells showed emission spectra with peaks varying between the peaks of adrenergic nerves (470 mμ) and enterochromaffin cells (525 mμ) (Fig 2) After HCl exposure, their fluorescence intensity decreased and spectral recording could not be obtained

The chemical analyses revealed that the glands contained 5 HT DA and NA (Table 1) As the table shows there were no obvious variations in the content of these monoamines between the young (8—10 weeks old) and the nearly adult (about 5 months old) animals Analyses of a larger material of newborn animals are necessary to evaluate whether the monoamine concentration differs from the older age groups as is indicated by the histochemical observations which showed richer occurrence of fluorescent cells in relation to exocrine parenchyma in the newborn than in the older animals The separate determinations of catecholamines in the duodenal and lienal part of the pancreas show that there is a tendency to a higher concentration of DA in the duodenal part (Table 2) The concentration of DA showed a fairly wide variation from animal to animal Such a variation was also found to occur when the tissue specimens from the different animals were compared histochemically

On the paper chromatogram two spots appeared with the same characteristics as authentic DA and NA respectively No dopa was detected in the pancreas of the newborn animals

Table 1

The concentration of NA DA and 5 HT in the pig pancreas

	8—10 weeks old animals µg/g M±SEM (n)	Appr 5 months old animals µg/g M±SEM (n)	Newborn animals µg/g
NA	0 18±0 0[illegible]5 (n=16)	0 31±0 063 (n=8)	—
DA	0 27±0 053 (n=16)	0 47±0 14 (n=8)	0 37 and 0 20
5 HT	0 33 and 0 25	0 28±0 034 (n=5)	0 79 and 0 51

Table 2

The concentration of DA and NA in the duodenal and the lienal halves of the pancreas from 8—10 weeks old pigs

Animal n o	Duodenal half		Lienal half	
	DA µg/g	NA µg/g	DA µg/g	NA µg/g
1	0 10	0 27	0 07	0 21
2	0 53	0 23	0 35	the sample lost
3	0 32	0 12	0 20	0 10
4	1 18	0 26	0 82	0 28
5	0 35	0 10	0 [illegible]1	0 14

DISCUSSION

The present investigation has shown the occurrence of NA DA and 5 HT in the pig pancreas

The content of pancreatic NA well corresponds to the number of adrenergic

nerves found Moreover, in sections treated with HCl vapor the fluorophore of these nerves behaved like that of NA containing sympathetic nerves in other tissues (Bjorklund et al 1968) Thus it seems reasonable to suppose that they contain NA rather than DA The distribution of these nerves was similar to that found in other mammalian species (Alm et al 1967)

The green fluorescent cells in the islets and scattered among exocrine cells had the same spectral characteristics as catecholamines Those having a sufficient fluorescence intensity to be analyzed in the microspectrograph after HCl treatment according to Bjorklund et al (1968) showed changes in their excitation spectra typical for DA Since the pancreas contains large amounts of DA and only three cell systems exhibited a green flourescence—i e adrenergic nerves, cells in ganglia, and an abundance of endocrine cells—it thus seems reasonable to suppose that the last mentioned cells contain DA The rarely occurring fluorescent cells in ganglia most probably contain a minute amount of the pancreatic DA since similar cells elsewhere in the autonomic nervous system seem to store DA (Bjorklund et al to be published)

The yellow fluorescent cells in the pancreatic ducts and scattered in the exocrine parenchyma showed spectral characteristics of 5 HT and some related substances (Caspersson et al 1966) Thus these cells probably contain at least part of the pancreatic 5 HT

As to the cell population in the islets and scattered in the exocrine parenchyma which displayed emission curves varying between those of catecholamines and 5 HT there are two possibilities which must mainly be taken into consideration they possibly contain 5 HT and DA in varying proportions or perhaps an unknown substance or substances capable of condensing with formaldehyde to a fluorescent derivative There is at present no definite proof of either of these possibilities It should be noted in this connection that the first mentioned alternative implying the remarkable phenomenon that two biologically active monoamines are stored in one and the same cell, is not merely an unfounded hypothesis there is strong support for the view that this is the case in the endocrine pancreas of the perinatal guinea pig Possibly there is a dual storage of these amines also in the adult guinea pig (Cegrell and Falck 1968) In any case, this investigation has demonstrated that the occurrence of DA in the endocrine pancreas is not a unique feature of the fetal and newborn guinea pig (Cegrell et al 1967) but that this amine occurs also in pig endocrine pancreas at least up to a late juvenile stage In fact investigations founded on microspectrometric methods suggest that DA is a commonly occurring monoamine in the mammalian endocrine pancreas (Cegrell 1968 a b) It should be emphasized in this connection that the identification procedure used in this study does not allow a strict classification of the cells exhibiting different spectral characteristics according to histologically definable types of endocrine cells a procedure for such identification which is currently under preparation necessitates *i a* the use of very thin sections This investigation has demonstrated big variations in the intensity of the cellular fluorescence reflecting big variations in the monoamine

content of the A_2 and B cells as can be expected if the monoamines have a functional role in the cells However from another point of view these variations are important to note they could imply that A and B cells at certain functional stages contain too small amounts of monoamines to be visualized histochemically which would introduce a difficulty in correlating the type of fluorescence to the type of islet cells

It is of great interest that many aldehyde fuchsin and silver negative cells which appeared intermingled with the B cells and thus (by definition) are A cells (see Hellerstrom et al 1964 Alm and Hellman 1964) exhibited a specific fluorescence A storage of monoamines in the A cells in the adult or nearly adult stage has so far been demonstrated only in the cat (Cegrell 1968 b) in the pre natal and newborn guinea pigs (but remarkably not in the adult guinea pig) the A cells seem to contain DA (Cegrell and Falck 1968) A recent comparative study (Cegrell 1968 a b, Cegrell and Falck 1968) shows that monoamines in the B cells have so far been observed only in the guinea pig and the human fetus In this study a specific fluorescence of the B cells in the pig have likewise been demonstrated this species is thus the only one so far found that has a monoamine store in both the A_2 and the B cells in the adult or nearly adult stage

The fluorescent cells which occur scattered among the exocrine cells represent a special problem in that their nature is not established Numerous similar cells have been found in the pancreas of the fetal and newborn guinea pig Postnatally however they rapidly disappear and in the adult stage they are found only occasionally A similar developmental pattern was registered in the pig where they were only rare at 5 month of age Some of these cells however probably represent true islet cells since occasional aldehyde fuchsin positive cells could be observed interposed between exocrine cells Moreover some of the scattered cells that displayed an intensity sufficient for microspectrographic analysis could be demonstrated to emit either a DA fluorescence or a fluorescence whose emission peaks varied between those of catecholamines and 5 HT i e the fluorescence types in endocrine cells of islets

The nature of the scattered cells with a clearly yellow fluorescence identical to that emitted by the enterochromaffin cells and which also appear in the adult guinea pig pancreas remains completely obscure

CATECHOLAMINES IN THE PANCREAS OF HUMAN FETUSES

by

Lennart Cegrell

A storage of monoamines has been revealed in the endocrine pancreas of many mammalian species (Falck and Hellman 1963 Cegrell et al 1964, 1968 Cegrell 1968 b) by means of the fluorescence method of Falck and Hillarp The presence of histochemically demonstrable monoamines at this site is not a general principle however and they were not observed in for instance the rat golden hamster, and cynomolgus monkey (Cegrell 1968 b) The aim of this study was to establish whether monoamines occur in the human endocrine pancreas In the pancreas of adult man Dayan (1967) could not demonstrate any catecholamines in the endocrine cells In a current investigation glands from patients more than 50 years old (5 cases) were obtained in association with operations on the pancreas and were analyzed in this laboratory The islets of Langerhans could readily be identified by the occurrence of reddish autofluorescent granules in the endocrine cells (see Hamperl 1934) After formaldehyde treatment some cells exhibited a very weak green fluorescence besides the autofluorescent granules It is difficult to ascertain however, whether that weak fluorescence derives from monoamines although it is not found in control specimens unexposed to formaldehyde No disturbing autofluorescence appears in the fetal pancreas and the present study was therefore performed on human fetuses made available in association with legal abortions

MATERIAL AND METHODS

The material consisted of pancreatic glands taken from 18 human fetuses gestational age 21 to 24 weeks Small tissue specimens were taken out as soon as possible post partum and analyzed according to the method of Falck and Hillarp (see General Methods)

RESULTS AND COMMENTS

In the tissue specimens from human fetal pancreas not treated with formaldehyde no autofluorescence was observed that could interfere with the specific fluorescence i e formaldehyde induced A specific green fluorescence developed

in adrenergic nerves and in islet cells The adrenergic nerves appeared as nerve bundles and as varicose terminals around vessels and scattered in the exocrine tissue The nerve bundles contained smooth as well as varicose fibres The number of fibres running among the exocrine acini was similar to that found in other species (Alm et al 1967) Adrenergic nerves were only sparsely found within the islets of Langerhans Groups of non fluorescent ganglion cells surrounded by a dense network of adrenergic terminals were found in the connective tissue

The islets of Langerhans contained many non fluorescent cells The intensity of the specific green fluorescence from the endocrine cells varied in the different fetuses from very weak barely visible to moderate, one and the same islet showed a slight variation between different cells These differences could reflect biological variations rather than variations introduced by technical errors as no parallel differences could be observed in the fluorescence intensity of the adrenergic nerves Similar individual differences have been found in fetal and newborn guinea pigs (Cegrell and Falck 1968)

In the microspectrographic analyses the adrenergic nerves and the green fluorescent cells showed the same spectral characteristics coinciding with those of catecholamines Because of the relatively low fluorescence intensity in most of the islet cells reliable microspectrographic results could not be obtained after treatment in HCl vapour except in the cells exhibiting a moderate fluorescence intensity In these cells the excitation spectra after HCl treatment changed in a way similar to that found in DA containing cells elsewhere whereas the fluorophore of the adrenergic nerves behaved similar to that of NA (cf Bjorklund et al 1968)

In three chemical determinations on human fetal pancreas (two determinations each on one gland one determination on five pooled glands) NA was found in a concentration of 0 37 0 43 and 0 47 $\mu g/g$ whereas no significant amounts of DA or A could be detected even when five glands were pooled

Judging from the histochemical microspectrographic, and chemical analyses there is no doubt that NA is stored in adrenergic nerves As to the endocrine cells there are reasons for believing that they store DA because the fluorescence in some cells behaves as DA after HCl treatment moreover similar results have been obtained in the endocrine pancreas of cat and dog and of the young pigmented rabbit and mouse and the presence of DA at this location has been established in the pig and guinea pig (Cegrell et al 1967 1968 Cegrell 1968 b) However for further chemical analyses to establish whether DA is present in human fetal pancreas larger amounts of pancreatic tissue are necessary

By comparing microphotographs showing the fluorescent cells the silver positive A_1 cells and the aldehyde fuchsin positive B cells from the same islet in one and the same section it was obvious that the fluorescent cells correspond to the B cells (Fig 4) So far no monoamine containing A cells have been found histochemically Further studies at other developmental stages of human pancreas will reveal whether monoamines are stored exclusively in the B cells in man If they are the human pancreas will form a pattern not so far found in

other mammalian species as monoamine are stored in both A cells and B cells in the pig and the perinatal guinea pig pancreas and are only found in the pancreatic A cells of the dog cat and the young pigmented mouse and young pigmented rabbit (cf Cegrell 1968 b)

So far no 5 HT containing yellow fluorescent cells have been found in human fetal pancreas neither as scattered cells in the exocrine tissue where they can be found in other species e g the pig and guinea pig (Cegrell et al 1967 1968 Cegrell and Falck 1968), nor as enterochromaffin like cells in the duct epithelium

ADRENERGIC NERVES AND MONOAMINE-CONTAINING CELLS IN THE MAMMALIAN ENDOCRINE PANCREAS

A COMPARATIVE STUDY

by

Lennart Cegrell

In recent studies on the occurrence of monoamines in the endocrine pancreas a storage of different types of monoamines was demonstrated in certain of the mammalian species examined (Falck and Hellman 1963 Cegrell et al 1964 Cegrell et al 1968) These findings suggest that generalities concerning functional mechanism might be forthcoming if data were available on the appearance of such amines in the pancreas of a wider variety of species and under a wider variety of physiological states This paper presents comparative aspects on the monoamine stores in the endocrine pancreas of further mammalian laboratory animals The occurrence of monoamines in the endocrine pancreas of man guinea pig and pig has been reported elsewhere (Falck and Hellman 1964 Cegrell and Falck 1968 Cegrell 1968 a Cegrell et al 1967 1968)

MATERIAL AND METHODS

The types and number of animals included in this study are listed in Table 1 Both sexes were used indiscriminately Small pancreatic specimens choosen at random from the entire gland were treated according *to the fluorescence method* of Falck and Hillarp (see General Methods)

When the islets of Langerhans contained no or only few fluorescent structures the islets could nevertheless be distinguished from the exocrine tissue because of their structural character and the arrangement of the vessels moreover in some animals the background fluorescence of the islets was somewhat different from that in the exocrine tissue In most cases this was enough to delimit the islets but in some cases it was necessary to make a comparison with consecutive sections stained in aldehyde fuchsin

Table 1

Mouse	adult young (5—15 days old) adult young (5—15 days old)	albino albino pigmented pigmented	25 12 21 15
Rat	adult young (7—15 days old)	albino pigmented	1[illegible] 3
Golden hamster	adult		14
Rabbit	adult young (10—20 days old) adult young (10—20 days old)	albino albino pigmented pigmented	8 4 4 8
Cat	adult young ([illegible]—6 week)		15 6
Dog	young (5 months) adult		1 6
Cynomolgus monkey	age unknown		5

RESULTS

In the tissue specimens no autofluorescence was present that could interfere with the observation of the specific i e the formaldehyde induced fluorescence except in the pancreatic specimens of some adult dogs (see below) In all species examined, a specific fluorescence was observed in nerve bundles and varicose adrenergic nerves which formed a dense network around larger vessels and occurred as scattered fibres in the exocrine tissue as noted in earlier observations (Alm et al 1967) Because of the great species differences in type and amount of monoamines visible in the islets of Langerhans and in cells scattered among exocrine cells as well as in islet nerves each species is described separately below The occurence of 5 HT containing (enterochromaffin) cells in the duct system will be reported in a forthcoming paper

Mouse

The varicose adrenergic nerve fibres when they occurred usually formed a wide meshed network around the islets and appeared scattered within the islets mostly associated with vessels sometimes a direct continuity was seen between the vascular nerve plexuses outside the islets and the islet nerve fibres This arrangement

was most obvious in large islets whereas in small islets only few or no fibres could be demonstrated

Generally the islets were devoid of fluorescent cells both in the albino mouse and the adult pigmented mouse though in some islets very few weakly green fluorescent cells could occasionally be seen In the islets of the young pigmented mouse however, some cells emitted a weak to moderate green cytoplasmic fluorescence The cells displaying the moderate fluorescence could be analyzed in the microspectrograph and showed excitation and emission peaks at 410 and 470 mμ respectively These spectra were identical with those of adrenergic nerves in the same section and those obtained from catecholamines in model systems under the measuring conditions used (cf Bjorklund et al 1968) After the sections had been treated in HCl vapor the excitation spectra changed in a way typical of DA At a comparison of the microphotos of the islet cell fluorescence with the microphotos obtained after staining the same sections with aldehyde fuchsin it was obvious that the fluorescent cells were aldehyde fuchsin negative (Fig 5)

Rat

The adrenergic nerves were distributed similar to those in the mouse No islet cells with a specific fluorescence have so far been observed in the albino animals In pigmented young rat only some few very weakly fluorescent cells were observed in the islets they were as rare as in albino mouse

Golden hamster

Of the mammalian species examined, the golden hamster possessed the most dense network of adrenergic nerves within the islets They appeared as varicose terminals forming peri insular and intra insular plexuses Several of them seemed to follow islet vessels and regular perivascular plexuses could be seen within the islets (Fig 6) The islet cells appeared dark in the fluorescence microscope only occasionally a few cells with a yellow fluorescence were found near or at the periphery of some islets The identity of these cells have not been established Their emission spectra were identical with that of 5 HT containing cells (enter ochromaffin cells see Erspamer 1954) in the gut

Rabbit

So far specifically fluorescent cells have been found only in young pigmented rabbit They appeared cattered in the exocrine tissue as green or yellow fluorescent cells and in the islets as green fluorescent cells often located peripherally (where the A cells are found see Ferner 1952) and emitting a weak to moderate intensity The moderately fluorescent cells showed excitation spectra typical for catecholamines which after HCl treatment changed in a way typical for DA The fluorescent cells were aldehyde fuchsin negative (Fig 7) Adrenergic nerves appeared only occasionally in association with the islets

Cat

The cat had more adrenergic nerves in the islets than the rat and mouse but less than the golden hamster The nerves surrounded small lobules of the endocrine cells and were sometimes clearly associated with the vessels (Fig 8) A few scattered cells morphologically resembling the islet cells emitting a light in the green to yellow range were found dispersed in the exocrine parenchyma The amount of such cells was higher in the young animals

The exact demarcation line between the endocrine and the exocrine parenchyma was hard to establish in its whole extension Nevertheless it could be seen that most of the islet cells were non fluorescent and that intermingled among these there were many cells displaying a specific fluorescence In the young animals most of the fluorescent cells emitted a green light whereas in the adults they showed either a green fluorescence or emitted a light in the green to yellow range The green fluorescent cells in adult and young animals exhibited the spectral characteristics of catecholamines after treatment in HCl vapor a shift appeared in the excitation spectra typical of DA The cells with the fluorescence in the green to yellow range displayed emission spectra whose maxima varied between that of a catecholamine and that of 5 HT A comparison of islets in microphotographs obtained first in the fluorescence microscope with those subsequently obtained after staining the same section with aldehyde fuchsin disclosed that the fluorescent cells did not stain in aldehyde fuchsin either in adult or in young animals

Dog

In the pancreatic islets of one five month old dog several cells emitting a light in the green to yellow range were intermingled with many nonfluorescent cells The emission spectra displayed peaks varying between that of a catecholamine and that of 5 HT In addition some cells exhibited a clearly green fluorescence and after HCl treatment the excitation spectra of these cells changed in a way typical of DA By comparing microphotos of the fluorescent cells with those of the silver positive A_1 cells and the aldehyde fuchsin positive B cells it was obvious that the monoamine store was located to the A cells (Fig 9) Scattered in the exocrine tissue were green fluorescent cells yellow fluorescent cells and cells with the intermediary fluorescence The islets received a rich supply of adrenergic nerves which formed perivascular plexuses or occurred as scattered fibres between the islet cells The uncinate process of this animal was not analyzed

In six adult animals both the uncinate process and the body of the pancreas were studied In agreement with earlier reports (Bencosme and Liepa 1955 Hellman et al 1962) the uncinate process was found to contain smaller islets than the body The islets were readily found because the islet nerves were distributed as in the above mentioned animal furthermore in some of the adult animals the islet cells contained reddish autofluorescent granules similar to those found in adult human pancreas (see Hamperl 1934) Few fluorescent islet cells were

found in the uncinate process In the islets of the body several cells exhibited a greenish fluorescence whose intensity varied widely from usually weak some times barely visible to moderate in a few cells In most of the cells the intensity was too low to make possible a reliable assessment of the emitted light Scattered in the exocrine tissue were many weakly green fluorescent cells in the uncinate process whereas only few such cells were found in the corpus In addition some clearly yellow fluorescent scattered cells were present both in the uncinate process and in the corpus

Cynomolgus monkey

No cells with a specific fluorescence could be seen in the islets or in the exocrine parenchyma The islets received very few adrenergic nerves

DISCUSSION

The amount of adrenergic nerves within the islets of Langerhans found with the fluorescence method varied widely between the different species The density of nerves was highest in the golden hamster where they form peri and intrainsular plexuses Many of these varicose fibres seem to be associated with vessels Fewer adrenergic nerves surrounding lobules of the islet cells were found in the dog and cat They were not always present in mouse and rat and only occasionally seen in the islets of monkey rabbit pig and guinea pig (cf Falck and Hellman 1963 1964 Cegrell and Falck 1968 Cegrell et al 1968) Using a cholinesterase staining method on the other hand Coupland (1958) demonstrated a rich supply of cholinergic nerve to the islets of Langerhans in cat rat and rabbit These nerves formed a dense peri insular network and a plexus of fibres following the vessels into the islets Similar results were obtained by Libman and Sutherland (1965) who studied the cholinergic innervation of the pancreatic islets in monkey cat rabbit guinea pig and rat It thus seems that most of the autonomic nerve fibres innervating the islets—at least in guinea pig rat rabbit and monkey—are cholinergic With the fluorescence histochemical method it could not be determined whether the adrenergic islet nerves innervated the vessels or the endocrine cells or both However they were sometimes found to form a plexus around islet vessels in a way similar to that found around vessels elsewhere in the body (cf Falck 1962 Norberg and Hamberger 1964) On the other hand a close relation between islet cells and autonomic fibres has been demonstrated in electron micro copic investigations (Bencosme 1959 Stahl 1963 Legg 1967 Esterhuizen et al 1968) which could indicate that the islet cells receive autonomic nerve fibres The great variation in the number of fluorescent nerves whether they represent an innervation of vessels and/or endocrine cells indicates that the adrenergic influence on the islets varies considerably in different species

With respect to the identity of the monoamine stored within islet cells however certain generalities can be stated Quite often there appears in the mammalian endocrine pancreas a primary catecholamine which is almost certainly DA Thus in the newborn guinea pig pancreas and in the pig pancreas (Cegrell et al 1967 1968), the presence of often high amounts of DA has been established by chromatographic and spectrofluorimetric methods In microspectrographic analyses green fluorescent islet cells in these species change their excitation spectra after HCl treatment as do DA-containing cells in other tissues or authentic DA in model systems (Bjorklund et al 1968) Further green fluorescent islet cells in the human fetus cat dog and the young pigmented mouse and rabbit show—upon HCl treatment—the same shift in their excitation spectra as the green fluorescent cells in the guinea pig and pig indicating that these cells also store DA In the fetal and newborn guinea pig pancreas, appreciable amounts of 5 HT have also been found and much argues for the view that this amine is stored in the endocrine part of the gland often in cells which in addition seem to contain DA These cells display in microspectrometric analyses emission spectra with peaks varying between that of a catecholamine and that of 5 HT (Cegrell 1967a, Cegrell et al 1967) probably implying a storage of varying proportions of the two amines *In the adult guinea pig microspectrometric analyses* have shown the B cell population to behave similarly Cells exhibiting such fluorescence characteristics have recently been found also in the pig (Cegrell et al 1968) and in the present study in the cat and dog These cells can store 5 HT and DA in varying proportions as in the guinea pig but it cannot yet be excluded that one or more unknown substances capable of condensing with formaldehyde to fluorescent derivatives are responsible for the emission curves obtained

As to the occurrence of monoamines in the mammalian endocrine pancreas in general there are certain peculiar features that should be considered In the guinea pig pronounced variations in concentration of the pancreatic monoamines which correlate to age and pigmentation have been established (Cegrell 1967b Cegrell and Falck 1968) Thus the content of 5 HT and DA in the endocrine pancreas is much higher perinatally than in adult life and much more DA is found in pigmented than in albino newborn animals In this study, only a restricted amount of fluorescent islet cells could be detected in the mouse and rabbit and then almost only in young pigmented animals Another finding suggesting that a decrease in endocrine pancreatic monoamines with increasing age is not an unusual phenomenon among mammals is that considerably more fluorescent cells emitting a fluorescence with the spectral characteristics of DA were found in the young cat and dog than in the adult animals This can be taken to indicate that the absence of histochemically demonstrable monoamines in adult specimens of mouse and rabbit is a quantitative rather than a qualitative feature which thus distinguishes them from other mammals whose endocrine pancreatic cells carry a sufficient amount of monoamines to be visualized with the fluorescence method Support for this suggestion came from a recent in

vestigation on the fate of exogenous dopa which indicated that monoaminergic mechanisms operate also in the cells of albino mouse (Cegrell 1968c) No specific fluorescence has so far been observed in islet cells of the cynomolgus monkey and hamsters young animals however have not so far been investigated Preliminary studies have shown that the cynomolgus monkey is similar to the mouse rat (Cegrell 1968c) and guinea pig (Cegrell and Falck 1968) to the extent that its islet cells can be rendered intensely fluorescent after administration of L dopa Readily demonstrable stores of monoamines in the endocrine pancreas have ear lier been reported in the duck guinea pig cat dog and horse (Falck and Hell man 1963) and exist also in the pig (Cegrell et al 1968) Also concerning the type of islet cell population storing demonstrable amounts of monoamines great species differences have been recorded Thus the specific fluorescence was con fined to A cells in the duck (Falck and Hellman 1963) in young pigmented mouse and rabbit, in cat and dog to the B cells only in the adult guinea pig and the human fetus and to A and B cells in the pig In the A_1 cells a specific fluorescence has so far been detected only in the duck (Falck and Hellman 1963) and has been shown not to appear in the guinea pig dog (one young animal) and human fetus

The nature of the fluorescent cells found scattered among exocrine cells in the pancreas of rabbit, cat and dog remains obscure Bencosme and Liepa (1955) found in the adult dog pancreas above all in the uncate process a type of cells appearing in the wall of ducts within the islets and scattered among the acinar cells which exhibited staining reactions deviating from that found in other pa renchymal cells, they designated these cells X cells In the 5 month old dog the fluorescent islet cells in the pancreatic body could be identified as A cells A_1 and B cells being non fluorescent However fluorescent cells appeared also in the islets of the uncinate process where no A cells have been found (Hellman et al 1962) nor any extractable glucagon (Bencosme et al 1955) It is tempting to suggest that the X cells of Bencosme and Liepa and the fluorescent cells in the uncinate process are the same cells a correlation that fails in one respect no fluorescent cells were found in the walls of the ducts

THE DEVELOPMENT OF MONOAMINE CONTAINING CELLS IN GUINEA-PIG PANCREAS

by

Lennart Cegrell and Bengt Falck

An occurrence of biogenic monoamines in the insulin producing B cells was first demonstrated in the adult guinea pig (Falck and Hellman 1964) Further studies have shown that the endocrine pancreas of guinea pig pre and postnatally differs considerably from that of adult animals with respect to the presence of mono amines Thus the A cells as well as the B cells in fetal and newborn guinea pigs possess a monoamine store (Cegrell 1967a) Histochemical evidence has shown that many endocrine cells contain a primary catecholamine at these develop mental stages and subsequent chemical and microspectrographic analyses dis closed the presence of DA often in high concentrations in the pancreas of new born animals whereas little or no DA was found in adult animals (Cegrell et al 1967) The concentration of DA varies widely in the newborn guinea pig pan creas and studies aimed at explaining these variations led to the unexpected find ing that the pigmented animals contain a significantly larger concentration of DA than albino newborn guinea pigs (Cegrell 1967b) Besides DA a storage of 5 HT was demonstrated in guinea pig pancreas and there is strong evidence that these two amines can occur concomitantly in some of the endocrine cells (Cegrell et al 1967 Cegrell 1967a)

This investigation was undertaken to study in more detail with histochemical and chemical techniques the development of monoamine containing cells from fetal up to adult stage

MATERIAL AND METHODS

The prenatal pancreatic material was obtained from 159 fetuses from 65 mo ther animals the fetal age varying from 15 days to about 65 days (full term) L dopa was administered intraperitonally (50 mg/kg) to 32 of the pregnant animals which were killed 30 minutes after the injection For further details see Table 1

Pregnant animals were left with a male guinea pig The day of delivery was counted as day one of the next gestation period This was checked by measuring

Table I

Fetal age (days)	Number of fetuses (number of pregnant animals within brackets)	
	untreated	L dopa treated
15—17	—	14 (8)
18—25	6 (2)	41 (15)
26—35	14 (5)	17 (8)
36—45	18 (5)	—
46—55	22 (10)	5 (1)
56—65	22 (11)	—

the length and weight of the fetuses (see Draper 1920) When the fetuses were younger than 30 days the abdominal part or alternatively the whole animals were freeze dried and serially sectioned in older fetuses the pancreatic glands could be excised The specimens were processed for fluorescence microscopy (see General Methods)

The postnatal pancreatic material was obtained from guinea pigs of either sex and of varying age 20 newborn (less than 24 hours old) 24 one day old (taken 24—30 hours after delivery) 16 two week old 5 one month old 5 two month old and 60 adult animals It was recorded whether the animals were pigmented or albino In the group of newborn animals four litters are included each consisting of one albino and two pigmented litter mates The animals were killed by decapitation and the pancreatic glands taken for histochemical and chemical analyses (see General Methods) Further the pancreatic glands from eight adult and three newborn albino animals treated with L dopa (50 mg/kg intraperitoneally) 30 minutes before killing were investigated histochemically In some of the experiments the recently developed silver nitrate procedure of Grimelius (1968) demonstrating the A cells was used

RESULTS

Histochemical analyses

Fetuses from untreated mother animals No specific (i e formaldehyde induced) fluorescence could be observed in the pancreatic cells before the age of 32 days In the 36—45 day stage monoamine containing cells and adrenergic nerves

could be readily demonstrated in the fetal pancreas At the beginning of this stage the fluorescent cells were few emitting a weak green light and often found in close contact with the duct system During the further development the number and intensity of green fluorescent cells increased and rather soon they appeared in the islets or scattered in the exocrine parenchyma Yellow fluorescent cells with a weak to moderate intensity also appeared at this stage and were most often found as scattered solitary cells There were great individual differences both concerning the number and the intensity of the fluorescent cells In the early part of this stage, only a sparse amount of adrenergic nerves were present and their number increased parallel with the increasing number of monoamine containing cells

At the 46—55 day stage a moderate to intense green fluorescence most often dominated in the islet cells and among the numerous fluorescent cells found scattered outside the islets individually or in small groups Within the islets the more strongly green fluorescent cells were often found in groups In both locations several cells emitted a light also in the green to yellow range A few of the cells scattered in the exocrine parenchyma exhibited a yellow fluorescence similar yellow fluorescence cells were occasionally also found within the islets In two albino fetuses the amount and the intensity of the green fluorescence were lower and most of the cells emitted a light in the green to yellow range

Comparison of microphotographs obtained in the fluorescence microscope with those obtained in the light microscope after subsequent staining with aldehyde fuchsin proved that both aldehyde fuchsin positive and aldehyde fuchsin negative cells were monoamine containing

In the pancreas of the 56—65 day old fetuses the cellular fluorescence was generally more intense and the picture was similar to that found in newborn animals

Fetuses from mother animals injected with L dopa No cells with a formaldehyde induced fluorescence could be observed until the 18 days stage From the 18th day on an epithelial bud growing out from the primitive gut could be observed it contained many cells displaying a strong green fluorescence appearing in the cytoplasm as well as in the nuclei These cells whose number increased with increasing age were dispersed among non fluorescent cells Early in the 26—35 day stage a large amount of green fluorescent cells were found mostly confined to the epithelium of the duct system they also appeared as discrete groups It was now evident that the fluorescence reflected an uptake of L-dopa into cells in the pancreatic anlage The arrangement of cells in islet formations was obvious in 33—35 days old fetuses although many fluorescent cells were still found scattered in the epithelial lining of the ducts (Fig 10) From this stage on an increasing amount of cells showed a fluorescence confined to the cytoplasm Five 50—60 day old albino fetuses after the L dopa injection showed a large number of fluorescent cells scattered in the exocrine parenchyma and in all or almost all the islet cells this fluorescence picture could not with certainty

be distinguished from the non L-dopa treated pigmented fetuses with the highest fluorescence intensity (see below)

Newborn and one day old animals The results obtained agreed with previous reports (Cegrell 1967a b Cegrell et al 1967) Thus a specific fluorescence was observed in adrenergic nerves, and in cells located in the islets and scattered in the exocrine parenchyma The distributional pattern of the adrenergic nerves was similar to that found previously (Cegrell et al 1967) The character of the emitted light of the fluorescent cells allowed a differentiation of three cell types clearly green fluorescent cells clearly yellow fluorescent cells and cells with a peculiar fluorescence in the green to yellow range The cells often showed long slender processes, most evident when they were isolated i e the scattered cells The fluorescence was often seen to be confined to cytoplasmic granules

Great individual variations with regard to the number and internsity of the green fluorescent cells were recorded As reported previously (Cegrell 1967b) there was generally an obvious difference between albino and pigmented animals the albino group having considerably less and usually weaker fluorescent cells than in the pigmented animals There was the same clear cut difference between the pigmented and albino animals even when they belonged to the same litter On the other hand after L dopa injection to newborn albino animals an intense green to yellow green cytoplasmic fluorescence was found in numerous cells scattered in the parenchyma and in almost all or all the islet cells i e the number of fluorescent pancreatic cells was similar to that of newborn pigmented animals

In conformity with earlier results (Cegrell 1967a) it was evident from the microspectrographic analyses that the exitation and the emission spectra of the green fluorescent cells were identical with those of the catecholamine fluo rophores and that the emission spectra of the yellow fluorescent cells agreed with that of the 5 HT fluorophore The cells with the peculiar fluorescence on the other hand showed emission spectra with peaks varying between those of cate cholamines and 5 HT When the sections were treated in HCl vapour according to the method of Bjorklund et al (1968) the spectra of the green fluorescent cells changed in a way typical of DA (cf Fig 1)

The islet cells displaying a clear and most often strong green fluorescence were usually found in groups sometimes forming caps upon those cells which emitted the intermediary fluorescence in the green to yellow range A comparison of colour micrographs obtained in the fluorescence microscope with those obtained after silver impregnation according to Hellman and Hellerstrom (1960) or after staining in aldehyde fuchsin showed that the green fluorescent cells correspond well to the aldehyde fuchsin negative cells and the green yellow cells to those stained in aldehyde fuchsin The silver positive (argyrophil) A_1 cells were found to be non fluorescent In the experiments where the silver impregnation of Gri melius (1968) was applied together with subsequent staining in aldehyde fuchsin the silver positive cells as well as the aldehyde fuchsin positive cells, were found to correspond to the fluorescent cells (Fig 12)

One and two week old animals Most of the fluorescent islet cells were of the

type emitting a light in the green to yellow range the number of green fluorescent cells in pigmented animals being clearly less than in pigmented newborn animals Some cells displaying a weak green light were often found in the periphery of the islets intermingled with non fluorescent cells Most of the fluorescent cells were identical with the aldehyde fuchsin positive cells but some were aldehyde fuchsin negative There were less fluorescent scattered cells here than in newborn animals

One month old animals There were less scattered cells in the exocrine parenchyma here than in the one to two week old animals They showed either a yellow fluorescence or the peculiar fluorescence in the green to yellow range and only occasionally were they clearly green fluorescent Almost all of the fluorescent cells in the islets were of the type emitting a light in the green to yellow range and were aldehyde fuchsin positive

Two month old and adult animals These two groups were very similar Only rarely were green fluorescent cells found in the islets which consisted mainly of cells emitting a fluorescence in the green to yellow range together with non fluorescent cells The former displayed emission spectra similar to those found in newborn animals i e the peaks varied between 470 mμ and 525 mμ The fluorescent cells whose intensity varied from weak to sometimes rather strong were identical with the aldehyde fuchsin positive cells this agrees with recent investigations (Falck and Hellman 1964) A few cells scattered in the exocrine parenchyma were regularly found most often emitting a moderate to sometimes strong yellow fluorescence The adrenergic nerves showed a distribution similar to that in the newborn animals

In the pancreas of the adult animals which were injected with L-dopa a strong yellow green cytoplasmic fluorescence developed in the islets and in many cells scattered in the exocrine parenchyma A few scattered cells still emitted a clear yellow light No non fluorescent endocrine cells within the islets could be observed A strong green fluorescence was now also seen in many cells located among the exocrine acini The appearance of these cells differed from that of the endocrine cells in several respects Their fluorescence was not confined to the cytoplasm but appeared also in the nuclei Further they were provided with one or more coarse or slender processes the slender ones sometimes reaching a length of two to three times the cell diameter The cell bodies and their processes were located in intimate contact with the exocrine acini Even in these L dopa injected animals most of the acinar cells were non fluorescent although in some areas there was a weak green diffuse fluorescence or fluorescent granules in the cells Thus no uptake of L dopa into exocrine cells in the guinea pig comparable to that found in the rat and mouse (Cegrell et al 1964 Alm et al 1967b) could be demonstrated

Chemical analyses

The results of the spectrophotofluorimetric determinations are given in Table 2 Obviously the concentration of NA is comparatively constant throughout the developmental stages included in these determinations This could be expected from the histochemical analyses which disclosed a fairly constant amount of green fluorescent nerves in the postnatal groups On the other hand the concentration of DA varied considerably between the groups these variations however corresponded well to the over all amount of green fluorescence found in the pancreatic cells at the different developmental stages

The differences in the concentration of pancreatic DA originally observed in pigmented and albino newborn guinea pigs from different litters (cf Cegrell 1967b) were obvious also when comparing litter mates Thus in three determinations each made on four pooled glands the pancreatic DA concentration was 0 17 μg/g in albino animals whereas pigmented litter mates showed a concentration of 1 61 and 3 17 μg/g

Reserpine treated animals histochemical and chemical analyses

Only newborn pigmented animals were included in this part of the investigation The chemical results are summarized in Table 3 Four hours after administration of reserpine (5 mg/kg subcutaneously) a pronounced depletion of the pancreatic NA had occurred whereas no significant decrease in DA could be recorded The DA figures lie within the range established in this and in a recent investigation on untreated newborn animals (Cegrell 1967b) In agreement with the chemical determinations only few weakly fluorescent varicose adrenergic fibres were found whereas the fluorescence of the pancreatic cells did not overtly differ from that found in untreated newborn animals Twenty four hours after the injection when no significant amounts of NA and a very low concentration of DA could be found the fluorescence picture had completely changed Now no adrenergic nerves could be observed and the fluorescent cells emitted a weak light in the green to yellow range (although their number was approximately the same as in untreated animals)

When the micrographs of the fluorescent cells were compared with those obtained after staining in aldehyde fuchsin it became obvious that both A and B cells were fluorescent as was the case in untreated animals The staining property of the B cells with regard to aldehyde fuchsin was the same in the two reserpine treated groups and did not differ from that in normal animals

DISCUSSION

The development of pancreatic islets in guinea pig fetuses was studied by Helly (1906) who found in the pancreatic anlage of 6 mm fetuses singular cells presumed to be primary islet cells and by Liegner (1932) who was able to demon

Table 2

The concentation of DA and NA in the guinea pig pancreas The determinations of new born animals (18 pigmented and 7 albino) recently reported (Cegrell 1967b) are included

Animals	DA μg/g M ± SEM (n)	NA μg/g M ± SEM (n)	Number of pooled glands
Newborn pigmented	1 74 ± 0 23 (20)	0 41 ± 0 013 (20)	4
albino	0 11 ± 0 019 (10)	0 39 ± 0 019 (10)	1
1 day old pigmented	1 92 ± 0 43 (7)	0 45 ± 0 031 (7)	4
1 week old pigmented	0 41 ± 0 16 (6)	0 36 ± 0 037 (6)	4
2 week old pigmented	0 33 ± 0 24 (4)	0 27 ± 0 013 (4)	4
1 month old pigmented	0 01 (1)	0 22 (1)	5
2 month old pigmented	0 03 (1)	0 33 (1)	5
adult pigmented	0 03 ± 0 005 (3)	0 43 ± 0 02 (3)	20

Table 3

The pancreatic concentration of NA and DA in newborn pigmented guinea pigs after treatment with reserpine Four glands were pooled for each determination There is a significant reduction of NA in the two groups ($p < 0\,001$) and of DA after 24 hours ($0\,001 < p < 0\,01$)

Reserpine treatment	NA μg/g M ± SEM (n)	DA μg/g M±SEM (n)
4 hrs	0 045 ± 0 0067 (5)	0 718 ± 0 116 (5)
24 hrs	0 007±0 0003 (5)	0 058±0 0033 (5)

strate islet formations in the 33 day but not in the 21 day stage Petersson (1966) using selective staining methods found pancreatic cells showing staining characteristics of A_1 and A cells in the 26 day old guinea pig fetus whereas no aldehyde fuchsin positive cells could be observed until the 39 day stages These results agree with those obtained by Hard (1946) who demonstrated A cells in the 30—33 and B cells in the 37—42 day stage In this study histochemically demonstrable amounts of monoamines were present from the 35 day stage on i e close to the developmental stage when the first aldehyde fuchsin positive cells were reported to occur In the following developmental stages there was an increase in the amount and intensity of fluorescent cells although considerable individual variations were noted and in agreement with an earlier report (Cegrell 1967b) the non pigmented animals usually displayed a clearly lower fluorescence than did the pigmented animals However when pregnant animals were

injected with L dopa it could be demonstrated that some cells in the pancreatic bud were fluorescent on L-dopa injection as early as the 18th day These cells possibly correspond to those described by Helly (1906) The study of succeeding developmental stages revealed that these cells appear first in the epithelium of the tubules and later in the islets or scattered in the exocrine parenchyma The formation of the first discrete groups of such cells was obvious from about the 26th day and from about the 33rd day typical islets were found in which most of the cells fluoresced It is thus possible to detect endocrine cells long before they acquire their special staining properties or their histochemically demonstrable stores of monoamines It should be noted in this connection that the A_1 cells too apparently take up L dopa since non fluorescent islet cells were not observed after the administration of this amino acid to 50—60 day old fetuses to newborn and to adult animals

There are reasons for believing that exogenous L dopa as such is not retained by endocrine cells in the pancreas (Cegrell 1968c) or in the thyroid (cf Falck and Owman 1968) but can be stored in the cells only after decarboxylation to a corresponding amine provided an intact storage mechanism is available in the cytoplasm The uptake experiments presented here showed that in the earlier stages the fluorescence induced by L dopa administration appeared in the cyto plasm as well as in the nuclei but that in the 26—35 day stage an increasing number of cells displayed a fluorescence confined to the cytoplasm Thus con sidering the intracellular distribution of the fluorophore it is conceivable that a storage mechanism for monoamines has developed at this stage i e. close to the first appearance of fluorescent endocrine cells demonstrated in the fetuses not explosed to L dopa

Up to and including the first postnatal days numerous cells emitting a light in the green to yellow green range and some emitting a clearly yellow light were found scattered among the exocrine cells Postnatally there occurred in this cell population a rapid decrease which could be registered already in one to two week old animals and was pronounced at the one month stage, in the adult pancreas few cells remained usually emitting a clear yellow fluorescence Previous studies in which other methods for visualizing the endocrine cell system in the guinea pig pancreas have been applied seem to agree with these findings Thus Bensley (1911) applied a supravital staining method and found in newborn animals numerous endocrine scattered cells which were no longer present in older ani mals Cavallero and Solcia (1964) obtained similar results in studies using dark field illumination whereby the refractile granules in the A cells can be visualized (Petersson et al 1962) Petersson (1966) using selective staining methods of islet cells reported the same distributional pattern in perinatal animals of all three cell types whereas in one month old animals the endocrine cells were almost exclusively confined to the islets At present the eventual fate of the scattered cells in the perinatal pancreas cannot be established The increasing volume of the exocrine pancreas can obviously not explain a diminution of their amount to such an extent Petersson (1966) reported that the proportion of the islet cells

decreases from 11—12 per cent of the pancreatic parenchyma in newborn animals to 4—7 per cent in one month old and older animals Factors such as degeneration of the cells or a continued migration into islets could be involved in the decrease It still remains to establish the identity of those relatively many scattered cells that attain a strong cytoplasmic fluorescence after administration of L dopa to adult guinea pigs and that in morphology and the ability to take up this amino acid are similar to the scattered cells found in young animals Do they represent a special cell population or are they endocrine cells which for some reason cannot be visualized with the staining methods used by the above mentioned authors? From the fluorescence histochemical point of view endocrine scattered cells could well be present in untreated animals without being detectable no A cells fluoresce in adult animals and the monoamine concentration of the B cells varies within wide limits (cf Falck and Hellman 1964) suggesting that even B cells can escape detection Grimelius (personal communication) has with his recently devised silver nitrate procedure (Grimelius 1968) found quite a few positive cells scattered among acinar cells in the pancreas of adult guinea pigs Preliminary studies in this laboratory have shown that many of the cells which fluorescence upon L dopa injection are identical with those staining according to Grimelius and further that occasionally aldehyde fuchsin positive cells can be found interposed among acinar cells It should be borne in mind that although Grimelius s impregnation stains the A cells in the human pancreas and the A cells of guinea pig islets (see Results) enterochromaffin cells also appear strongly positive Further identification experiments and specificity tests are thus necessary for eventually clarifying the nature of the mentioned cells

This and a recent report (Cegrell et al 1967) have demonstrated that three monoamines are present in the guinea pig pancreas namely NA DA and 5 HT There are reasons for believing that the NA measured chemically is located in adrenergic nerves (Cegrell et al 1967) which are histochemically found mainly around vessels and running among the exocrine acini (cf Alm et al 1967) whereas DA and 5 HT are located extraneuronally Further strong support for this view is presented in this study in short term experiments reserpine caused an almost complete disappearance of the pancreatic NA leaving the DA almost unaffected concomitant histochemical analyses displayed a pronounced decrease in the nervous fluorescence only In agreement with a recent investigation (Cegrell et al 1967) a striking correlation has been demonstrated between on one hand the amount and intensity of cells exhibiting a green fluorescence (which has the characteristics of a primary catecholamine) and on the other the concentration of pancreatic DA This correlation was even more obvious when comparing the pancreas of newborn pigmented and albino guinea pigs the former group containing many more green fluorescent cells and DA in the pancreas than the latter group (Cegrell 1967b) These results have been confirmed in this study which in addition has established that in postnatal life there is a parallel decrease in the amount of green fluorescent cells and pancreatic DA with increasing age Further 24 hours after the administration of reserpine a dramatic

decrease had occurred in both the green fluorescence and the content of DA Moreover when the recently developed method for the differentiation between the fluorophores of the two primary catecholamines (Bjorklund et al 1968) was applied to green fluorescent cells they *showed in the microspectrograph the* characteristics of DA Thus at least most of the pancreatic DA is located in cells displaying a specific fluorescence

The pancreatic 5 HT which has been demonstrated chemically in 50 day old fetuses and older animals (Cegrell et al 1967) is at least up to two weeks post natally only to a minute extent located in enterochromaffin cells as shown by the fact that a specific indole fluorescence in the pancreatic ducts only appears later in the development (Cegrell et al 1967 and unpublished observations) This and other findings (Cegrell et al 1967) support the view that the bulk of 5 HT is stored in specifically fluorescent cells occurring in the islets and scattered among the exocrine parenchyma Some of these cells in fact exhibit an emission spectrum identical with that of enterochromaffin cells It seems however that part of the 5 HT is stored—together with DA—in those cells that display the peculiar fluorescence in the green to yellow range (see Cegrell 1967a Cegrell et al 1967) One evidence justifying this assumption is that these cells in the microspectrograph *show emission spectra whose maxima vary between those of* catecholamine and 5 HT a phenomenon that rather reflects a concomitant occurrence of 5 HT and DA in varying proportions than a storage of some other substance or substances capable of forming upon formaldehyde exposure fluorophores that give rise to such varying spectra The results of the extended micro spectrometric analyses of the cells with intermediary fluorescence presented in this study completely agreed with those earlier reported (Cegrell 1967a)

The monoamine fluorescence seems to be confined to cytoplasmic granules an observation that has been confirmed in preliminary studies of sections having a thickness of 2—3 μ (Cegrell and Falck to be published) The relation between these granules and those believed to store the insulin or insulin precursor is not at present known It should be noted that there are no overt differences in the number of aldehyde fuchsin positive granules in the B cells of normal animals and of animals depleted of their pancreatic monoamines by reserpine treatment

Hellman and Falck (1964) identified the fluorescent islet cells in the adult guinea pig pancreas as B cells this study confirms it However in the perinatal material a specific fluorescence is undoubtedly displayed also by A cells but not by the silver positive A_1 cells The formation of mantle islets and bipolar islets which implies that the A cells occur as peripheral cell strands around the B cells or as separate parts of the islets (see Ferner 1952 Petersson 1966) could be observed in the fluorescence microscope islet cells emitting a clear and most often strong green light were arranged in a pattern similar to the A cells and as expected they could not be demonstrated with aldehyde fuchsin or the silver impregnation according to Hellman and Hellerstrom (1960) After the HCl treatment these cells together with many scattered cells in the parenchyma changed their spectral characteristics in a way typical of DA Thus DA is stored

in the A cells a finding in conformity with the observation that the fluorescence in the A cells has disappeared in the adult stage when also the pancreatic DA has decreased to a very low level (see Table 2)

There is still to be established the biological significance of the great differences in the monoamine concentration of the pancreatic endocrine cells which have been verified to occur on the one hand, between guinea pigs of different ages and on the other between pigmented and albino perinatal animals Concerning the last mentioned animals the difference does not seem to be a question of strain disparity since the difference was evident also in litter mates Furthermore a similar situation has been demonstrated in the mouse and rabbit (Cegrell 1968b) It seems impossible to explain the difference by dissimilarities in amount of pancreatic cells when fetal (50—60 day old) and newborn albino animals were treated with L dopa there could be noted in a number of endocrine cells an uptake by no means less than that found in pigmented animal of corresponding age Such a difference is not found exclusively in the endocrine pancreas in young pigmented—but not in albino mice—there has been demonstrated a considerable store of DA in cutaneous mast cells (Cegrell et al 1967b)

UPTAKE OF L-DOPA IN THE PANCREATIC ISLETS OF ALBINO MICE

by

Lennart Cegrell

By means of the fluorescence method of Falck and Hillarp a storage of biogenic monoamines has been demonstrated in the endocrine cells of the pancreas in many mammals although in some species only at early stages of development (Cegrell 1968b) However, in the cynomolgus monkey, rat golden hamster and the albino variants of rabbit and mouse no biogenic monoamines have so far been detected in the islets of Langerhans It is not yet known whether this finding reflects a principal difference or merely indicates that the islet cells operate with monoamines in very low concentrations or perhaps with other types of monoamines that have functional properties similar to those visualized histochemically in certain species The last mentioned alternatives imply that the compounds cannot be detected with the histochemical methods so far available

An uptake into mouse islets of the amino acids the immediate precursors in the biosynthesis of monoamines (L dopa and 5 HTP) has recently been demonstrated (Cegrell et al 1964 Ritzen et al 1965 Gershon and Ross 1966) In the guinea pig a storage of monoamines has been shown in the islets of Langerhans (Falck and Hellman 1964) and the endocrine cells are able to take up L-dopa This uptake ability can be demonstrated in fetal life long before the monoamines themselves appear, and it persits into the adult stage also in those cells which then contain no demonstrable amounts of monoamines (Cegrell and Falck 1968)

The aim of the present investigation has been to elucidate whether this uptake implies a monoamine mechanism in the islets cells or is part of a more general amino acid uptake

MATERIALS

Pancreatic glands from 118 adult albino mice were analyzed The animals were injected with the different substances shown in Table 1 each group consisted of at least four animals Dopa and DA were injected intravenously in a tail vein whereas the monoamine-oxidase inhibitor nialamide (Niamid Pfizer) (Beil et al 1964) and the decarboxylase inhibitors NSD 1015 (m hydroxybenzyl hydra

Table 1

Group	*Treatment*
1	L dopa 40 mg/kg (10 and 30 min 1 2 and 4 hrs)
2	D dopa 40 mg/kg (10 min 1 and 2 hrs)
3	DA 40 mg/kg (10 min and 1 hr)
4	DA 100 mg/kg (10 min and 1 hr)
5	Nialamide 100 mg/kg (1 hr) + L dopa 40 mg/kg (10 min 2 4 and 6 hrs)
6	Nialamide 100 mg/kg (1 hr) + DA 100 mg/kg (10 min 1 and 2 hrs)
7	NSD 1015 100 mg/kg (1 hr) + L dopa 40 mg/kg (10 and 30 min and 1 hr)
8	Ro 4 4602 100 mg/kg (1 hr) + L dopa 40 mg/kg (1 hr)

zine Smith and Nephew through Ferrosan AB Sweden) (Carlson 1964) and Ro 4 4602 (N (DL seryl) N 2 3 4 trihydroxybenzyl hydrazine Hoffman La Roche) (Pletscher and Gey 1963) were injected intraperitoneally The animals were killed by decapitation and a piece from any part of the pancreas was im mediately excised and processed for the fluresence method of Falck and Hillarp (see General Methods)

RESULTS AND COMMENTS

In normal untreated animals a specific (i e formaldehyde induced) fluores cence was found in adrenergic nerves in the exocrine tissue mostly confined to the vessels this agrees with earlier results (Alm et al 1967) Adrenergic fibres in connection with islets had the same distribution as described elsewhere (Cegrell 1968b)

As early as 10 minutes after the injection of L dopa a cytoplasmic fluorescence could be seen in islet cells and the fluorescence intensity varied from moderate to sometimes rather high throughout the islets Non fluorescent cells were not with certainty observed *It can therefore be concluded that the fluorescence was* located in both A cells and B cells Since in the mouse islets the A cells are located in the periphery and the B cells in the central part (see Ferner 1952) these dif ferences in the fluorescence intensity appeared in both cell groups Thirty and sixty minutes after the injection of L dopa the islet cells showed the same degree of fluorescence as after 10 minutes After two hours the fluorescence intensity had decreased in many cells although some mostly located in the periphery of the islets still showed a moderate to rather high fluorescence intensity Even after four hours some weakly to moderately fluorescent cells remained in the islets

These findings indicate that the fluorescence persists for a longer time in the A cells than in the B cells Consecutive staining with aldehyde fuchsin revealed that most of the fluorescent cells with the higher intensity and found at the periphery of the islets two and four hours after the L dopa injection were not aldehyde fuchsin positive

The animals pretreated with the potent monoamine oxidase inhibitor niala mide showed a fluorescence of high intensity in almost all the islet cells as early as ten minutes after the L dopa injection No obvious decrease in the number of the fluorescent cells and their fluorescence intensity could be seen two hours after the L dopa injection (Fig 13) Four hours after the injection some cells often with a peripheral distribution in the islets showed a moderate intensity which had further decreased two hours later (Fig 14)

Thus the animals in which monoamine oxidase had been inhibited with nia lamide had a fluorescence of a somewhat higher intensity and a longer duration than when L dopa was given alone This indicates that L dopa has been taken up by the islet cells and has then apparently been decarboxylated to a catechol amine since dopa itself is not known to be a substrate for monoamine-oxidase Moreover the results show that monoamine oxidase is involved in the break down of monoamines in the islet cells This agrees with the results obtained by Petkov (1965) who using the tetrazolium method demonstrated monoamine oxidase activity in the islets of many species

The suggested monoamine synthesis in the islet cells is further supported by the experiments in which the animals were pretreated with the dopa decarboxy lase inhibitors One hour after the injection of L dopa to animals pretreated with Ro 4 4602 at most a weak fluorescence was observed in the islet cells On the other hand after pre reatment with NSD 1015 instead of Ro 4 4602 most of the islet cells emitted a moderate cytoplasmic fluorescence one hour after the L dopa injection The reason for this discrepancy is perhaps that Ro 4 4602 still—and even two hours after its administration—efficiently inhibits decarboxylation whereas the action of NSD 1015 has already begun to deteriorate This was supported by the finding that when tissues were taken within a shorter time after injection of NSD 1015 (10 and 30 minutes after the administration of L dopa) the islet fluorescence was essentially the same as in the Ro 4 4602 experiment Thus a fluorescence accumulation of L dopa in the islet cells obviously occurs only if conditions exist for a decarboxylation of the amino acid to the correspond ing monoamine

However when the animals were injected with the monoamine itself—i e DA —no fluorescence could be demonstrated in the islet cells even at a high dose level This might be due to a rapid elimination of DA from the blood stream thus decreasing the amount of DA available for the islet cells Therefore in another series animals were pretreated with nialamide in order to reduce the breakdown of injected DA Even under these severe conditions only a slight fluorescence appeared in the islet cells These experiments strongly suggest that most—if not

all—of the monoamine present in the islet cells after a L dopa injection has been synthesized within these cells

Administration of D dopa produced no fluorescence in the endocrine cells This indicates that the amino acid uptake is stereo specific (cf Gibson and Wiseman 1951, Adams Ray et al 1963 Bertler et al 1966 Larsson et al 1966) although it cannot be excluded that the absence of cellular fluorescence is due to the inefficient decarboxylation of D dopa (Holtz et al 1939 Freter et al 1957) possibly entering the cells

Taken together the experiments show that L dopa can be taken up into the islet cells, possibly by a stereo specific mechanism and then decarboxylated to a monoamine stored in the cytoplasm which can be metabolised by monoamine oxidase Judging by the fluorescence studies there seems to be no certain difference between the A cells and the B cells except that the fluorescence persists for a longer time in the A cells which agrees with the absence of monoamine oxidase activity in these cells (Petkov 1965)

Cells which appear scattered among the exocrine cells and with morphology and uptake ability of L dopa similar to the islet cells have been reported to occur in the guinea pig pancreas (Cegrell and Falck 1968) Such extra insular cells could not be detected in any of these experiments Since in the mouse pancreas the acinar cells efficiently take up L dopa (Cegrell et al 1964 Alm et al 1967b) it is possible that any endocrine cells within the acini will be masked by the intense fluorescence of the exocrine cells It is less likely however, that any significant amount of such cells exist since in the one hour experiments large exocrine areas exhibit only a relatively weak fluorescence whereas the islet cells still fluoresce as strongly as after 10 and 30 minutes

Similar results were obtained when the animals were injected with L 5 HTP and 5 HT instead of L dopa and DA respectively (unpublished observations) By using an autoradiographic technique Ritzen et al (1965) demonstrated an uptake of labelled 5 HTP in most of the islet cells 10 minutes and four hours after the injection Similarly, using an autoradiographic technique Gershon and Ross (1966) could demonstrate signs of radioactivity in the B cells of the pancreatic islets up to 24 hours after the injection of labelled 5 HTP

After an injection of labelled L dopa an activity was found in some peripherally located islet cells possibly A cells, one and twenty four hours after the injection (Hammarstrom et al personal communication) Comparison of these results with those obtained with the histochemical method in this study makes it obvious that labelled material can be demonstrated for a longer time than the specific fluorescence It should be remembered that the autoradiographic technique has a completely different degree of sensitivity than the fluorescence method although it cannot be excluded that the radioactivity is bound to a metabolite of 5 HTP or L-dopa not condensing with formaldehyde to fluorescent products

The present results point to the improbability that the uptake of L dopa can be regarded only as part of a more general or unspecific amino acid uptake into the islet cells On the contrary they indicate that the investigated amino acids

participate in a monoaminergic mechanism in the islet cells and that the stores of monoamines are too low to allow histochemical demonstration This is supported by the finding that DA occurs in the islets only of young pigmented mice whereas adult albino mice contain at most some few weakly green fluorescent cells (Cegrell 1968b) These differences between pigmented and albino animals have also been found in the guinea pig (Cegrell 1967b) and rabbit (Cegrell 1968b) again indicating that the absence of a specific fluorescence in the endocrine pancreas is a quantitative rather than a qualitative feature It should be emphasized however, that so far it cannot be excluded that some other amine corresponding to the monoamines found in other mammals operates in the islets of the mouse Together with the findings of similar situations in the system of enterochromaffin cells in the gastric mucosa (Hakanson et al 1967) and the calcitonin producing cells of the thyroid (Larsson et al 1966) the present results emphasize that the absence of catecholamines or 5 HT in certain endocrine cell systems by no means excludes the possibility that monoamine mechanisms operate in such cells This is supported by the fact that the monoamines can be demonstrated in such cell systems of some species (Falck and Owman 1968)

GENERAL SUMMARY

The occurrence of certain biogenic monoamines (5 HT, DA, NA and A) in the endocrine pancreas of some mammalian species was studied in the present investigation. Chemical methods and the fluorescence histochemical method for visualizing biogenic monoamines combined with microspectrometric procedures *i.a.* allowing a differentiation between DA and NA were used. The material comprises human fetus, cynomolgus monkey, pig, dog, cat, rabbit, guinea pig, golden hamster, rat and mouse.

The distribution of NA containing adrenergic nerves in the islets of Langerhans showed considerable species differences. Thus, a dense network of varicose fibres appeared in the islets of the golden hamster, a somewhat less amount in the dog and cat, whereas only occasional fibres were present in the other species. Many of these nerves seemed to follow islet vessels but there were fibres running among endocrine cells without apparent contact with vessels. On the other hand the adrenergic nerves in the exocrine parenchyma showed a similar distributional pattern in all species studied.

There was no chemical or histochemical evidence for the presence of A in the pancreas of any of these species.

A storage of monoamines in endocrine cells was demonstrated in the human fetus, pig, dog, cat and guinea pig, but not in the cynomolgus monkey, golden hamster and rat. In the islets of rabbit and mouse a monoamine store could be demonstrated but only in young pigmented animals.

In the guinea pig pancreas—most extensively studied in the present investigation—a specific monoamine fluorescence was displayed by numerous cells in the islets or scattered among acinar cells in the perinatal period. The emitted light was either green (indicating a catecholamine) or yellow (indicating a tryptamine) or had a more ill definable colour in the green to yellow range. Chemically, DA and 5 HT were found to be present, often in high amounts, and there was strong evidence for the view that both those amines are stored in the endocrine pancreas. The cells displaying a fluorescence in the green to yellow range showed emission spectra with peaks varying between the emission maxima of catecholamines and 5 HT. Probably these cells contain both DA and 5 TH in varying proportions. The number of cells exhibiting a strong green fluorescence as well as the pancreatic content of DA was much higher in newborn pigmented than in newborn albino animals.

During the postnatal period the pancreatic DA decreases in parallel with a decrease in the amount and intensity of cells with green fluorescence. In the adult stage by far most of the monoamine containing cells showed an intermediary

fluorescence in the green to yellow range and thus possibly store DA and 5 HT concomitantly

In the fetal guinea pig pancreas the cells displaying a specific fluorescence could be seen from about the 35 day stage Somewhat later they constituted the dominant population in the islets and were found in rich amounts as single cells interposed between acinar cells However in experiments where L dopa was administered to mother animals the endocrine cells could be readily demonstrated by means of the fluorescence method from the 18th day onwards

In the pig pancreas DA and 5 HT were likewise found by chemical analyses A monoamine fluorescence was displayed by most of the islet cells and by cells scattered in the exocrine acini Some of these cells showed the fluorescence characteristics of DA whereas others emitted a light in the green to yellow range with emission peaks varying between those of catecholamines and 5 HT A clearly yellow fluorescence was found in some of the scattered cells and in rare cells occurring in the epithelial lining of the pancreatic ducts The intermediary fluorescence can reflect a dual storage of DA and 5 HT or the presence of an unknown substance or substances capable of forming a fluorphor with formaldehyde

Islet cells displaying a similar intermediary fluorescence were also found in cat and dog

In the endocrine pancreas of the human fetus cat dog young pigmented rabbit and mouse were cells emitting a fluorescence indicating the presence of DA

It was concluded that DA seems to be a commonly occurring amine in the mammalian endocrine pancreas and there is evidence for the opinion that it is present in higher amounts in young than in adult animals

The monoamines were detected in the A_2 and B cells of pig and perinatal guinea pig in the B cells of the human fetus and adult guinea pig and in A cells of cat young pigmented rabbit and mouse In one 5 month old dog the A cells displayed monoamine fluorescence

The absence of histochemically demonstrable amounts of monoamines in islet cells does not necessarily rule out the operation of monoaminergic mechanisms in such cells Thus evidence was produced showing that L dopa—but not D dopa—administered to the albino mouse is taken up by the islet cells and then probably decarboxylated to a monoamine stored and metabolized by monoamine oxidase in the cytoplasm A similar mechanism was also found in the A cells of the adult guinea pig

The present investigation has demonstrated that biologically very active and therefore important monoamines occur in definible endocrine cell systems in the mammalian pancreas suggesting the possibility that they are of functional importance for the homone producing cells

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FIGURES

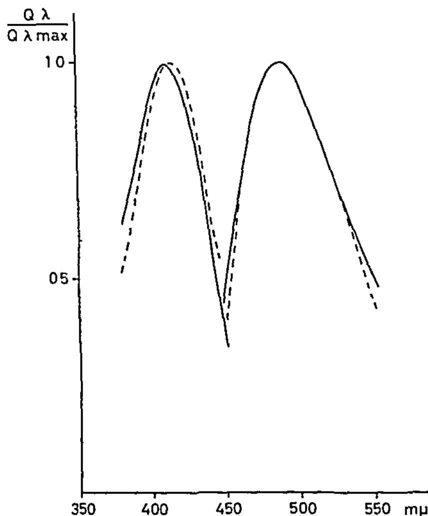


Fig 1 Excitation and emission spectra obtained from green fluorescent cells ( ) and adrenergic nerves (————) in the pig pancreas After HCl treatment there is a shift in excitation spectra of green fluorescent cells ( )

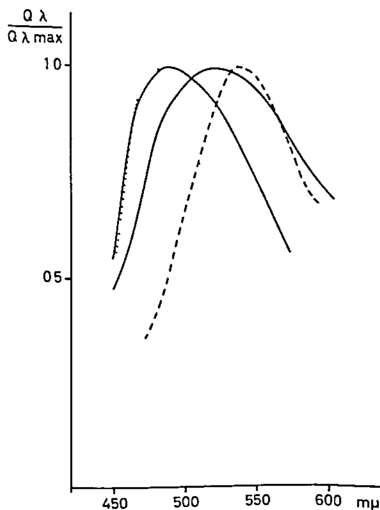


Fig 2 Emission spectra obtained in the p g pancreas from green fluorescent cells ( ) yellow fluorescent cells ( ) and from two different cells with the fluorescence in the green to yellow range (————)

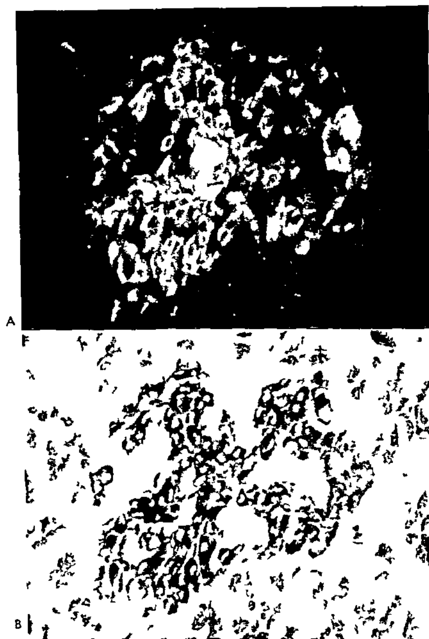


Fig 3 Specific fluorescence in most of the islet cells in a 8—10 week old pig A few adrenergic varicose nerve fibres are seen within the islet (A) The same islet after staining with aldehyde fuchsin (B) Most or perhaps all of the aldehyde fuchsin positive cells belong to the population of fluorescent cells Some aldehyde fuchsin negative cells are also fluorescent ×365

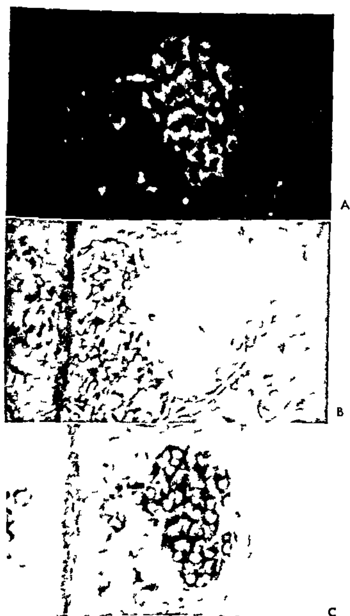


Fig 4 An islet of human fetus as seen in the fluorescence microscope (A) and after subsequent silver impregnation for A_1 cells (B) and aldehyde fuchsin staining (C) The fluorescent cells are identical with the aldehyde fuchsin positive cells. The A cells and B cells are well separated forming so-called bipolar islets (Ferner 1952 Robb 1961 Bjorkman et al 1966) ×245



Fig 7 Fluorescent peripherally located islet cells from a pigmented young (18 day old) rabbit (A) the aldehyde fuchsin cells being assembled in the central part (B) The fluorescent cells are aldehyde fuchsin negative ×384

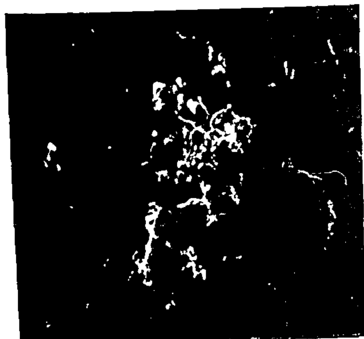


Fig 8 A pancreatic islet of a cat with fluorescent adrenergic nerves surrounding small groups of endocrine cells some of which show a monoamine fluorescence ×200



Fig 9 An islet of Langerhans in a 5 month old dog The fluorescent cells (A) the silver positive A1 cells (B) and the aldehyde fuchsin positive B cells (C) The fluorescent cell correspond to neither the A1 cells nor the B cells ×262



Fig 10 The pancreatic anlage of a 35 day old guinea pig fetus whose mother w
with L-dopa Intensely fluorescent cells can be seen in the islet formations and
duct epithelium ×?22

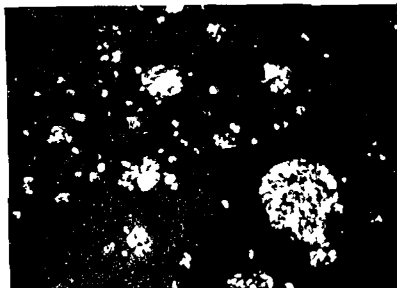


Fig 11 A section from the pancreas of a pigmented newborn guinea pig Islet cells and numerous cells scattered in the exocrine parenchyma are seen displaying a varying fluorescence intensity X140

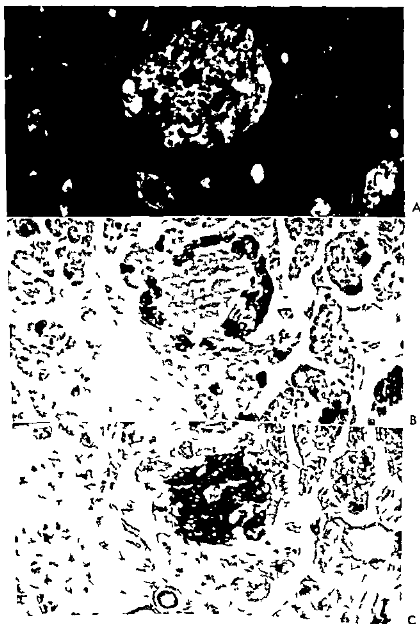


Fig 12 Fluorescent cells in the pancreas of a new born pigmented guinea pig (A) silver positive cells according to the method of Grimelius (1968) (B) and aldehyde fuchsin positive cells (C). It can be clearly seen that the silver positive A cells and the aldehyde fuchsin positive B cells show a specific fluorescence ×193



Fig 13 The intense cytoplasmic fluorescence displayed by the islet cells in a nialamide pretreated mouse killed two hours after the L dopa injection ×352





A B

Fig 14 An islet in a malamide pretreated mouse killed four hours after the L dopa injection Fluorescent cells (A) and aldehyde fuchsin positive cells (B) Most of the cells showing a high fluorescence intensity are aldehyde fuchsin negative ×256

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 315

NUCLEIC ACIDS IN EXPERIMENTAL GRANULOMA

BY

JUHANI AHONEN

TURKU 1968

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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY UNIVERSITY OF TURKU
TURKU FINLAND

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ERRATA

Fig 3 on page 18
for *"Sediment P III microsomal fraction"* read
'Sediment P II mitochondrial fraction'

On page 30, first row
for ' and 28 2S' read ' and 27 9S"

On page 54, legend for Table 11, first row
for 'RNA was' read "RNA were'

On page 57, Table 14 column AMP last row
for "29 9' read '20 9"

On page 72
for "Wodssner Jr' read 'Woessner Jr'

CONTENTS

Turku Kirjapaino Polytypos 1969

INTRODUCTION

The formation of granulation tissue is a basic phenomenon in mammalian repair processes for instance in the healing of wounds After the initial inflammatory reaction has subsided the space between wound margins is filled with fibroblasts Their activity results in the deposition of collagen and ground substance which transforms the vascular granulation tissue into an avascular scar During a relative short time there occur developmental phases of proliferation, differentiation of the cells for a specialized function and involution

The molecular mechanisms regulating fibroblast activities during the development of granulation tissue are unknown Present day concepts emphasize the role of nucleic acids in directing cellular functions The necessary information for metabolic processes is contained in the nucleotide sequences of deoxyribonucleic acid (DNA) Complementary ribonucleic acids (RNA) carry this information to the cytoplasmic sites of protein biosynthesis where other RNA have important functions

Nucleic acids in granulation tissue have been little studied The emphasis in previous investigations has been on the contents of DNA and RNA at different phases of tissue development In this work the methods of extraction and fractionation of nucleic acids have been adapted to experimental granulation tissue produced by subcutaneous implantation of viscose cellulose sponges The capacity of the tissue to synthesize nucleic acids and proteins including collagen was measured during its development The properties of DNA and of various RNA fractions were studied to determine whether changes in the properties of some nucleic acid fractions are associated with various phases of development of granuloma tissue

ABBREVIATIONS

C cytosine
A adenine
U uracil
G guanine
T thymine
CMP AMP UMP, GMP
2 and 3 monophosphates
of cytidine adenosine, uridine,
guanosine
LMW RNA RNA of low molecular
weight, soluble in 3 M sodium acetate
solution
HMW RNA RNA of high molecular
weight insoluble in 3 M sodium acetate
solution

mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
DOCA	sodium deoxycholate
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
PCA	perchloric acid

INTRODUCTION

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REVIEW OF THE LITERATURE

Production of experimental granulomas

Several methods have been used to induce the formation of granulation tissue experimentally The most suitable method has proved to be the implantation of synthetic (Grindlay and Waugh 1951 Gilmer Tooms and Salvatore 1961 Woessner et al 1961) and semisynthetic sponge like materials (Viljanto and Kulonen 1962 Viljanto 1964) The granulation tissue formed in the sponge is similar both histologically and chemically to that formed in a healing wound (Boucek and Noble 1955 Viljanto and Kivikoski 1962 Viljanto and Kulonen 1962 Viljanto 1964) The amount of tissue formed is positively correlated with the size of the sponge (Boucek and Noble 1955)

The formation of granulation tissue has been induced in guinea pigs also by subcutaneous injections of carrageenin (Robertson and Schwartz 1953) The granulomas have been used to study the mechanism of collagen synthesis (Robertson et al 1959 Green and Lowther 1959, Chvapil and Cmuchalova 1960) A carrageenin granuloma however, is less suitable for the study of developmental phases of granulation tissue since it is rapidly resorbed (Robertson and Schwartz 1953 Viljanto et al 1962)

Nucleic acids in granulation tissue

Deoxyribonucleic acids

Woessner and Boucek (1961a) found that the content of DNA in granulomas formed in polyvinyl (Ivalon) sponges implanted in rats increases up to the 20th day of development and then decreases slightly Bollet et al (1958), who studied similar granuloma found the amount of DNA to be a maximum on the 14th day Viljanto (1964) reported the amount of DNA per sponge to be maximal in implanted viscose cellulose sponges on the 21st day and Lampiaho and Kulonen (1967) observed the maximal amount of DNA on the third week of granuloma development The maximum amount of DNA found by Woessner and Boucek in Ivalon sponge implants was 1 8 mg per 100 mg whereas Viljanto reported 4 0 mg per 100 mg and Lampiaho and Kulonen 2 9 mg per 100 mg in viscose cellulose implants Bashey Woessner and Boucek (1964) observed that the amount of DNA per sponge was higher in male than in female rats

Williamson and Guschlbauer (1961) and Guschlbauer and Williamson (1964) studied the DNA content and the rate of incorporation of radioactive phosphate into DNA in regenerating wound tissue The amount of DNA per mg of tissue nitrogen including the nitrogen of collagen rose to a maximum on the 7th—9th day and then

subcellular fractions obtained by differential centrifugation of wound tissue homogenates The nuclear fraction contained 50 % the microsomal fraction 10 % and the nonparticulate fraction 45 % of the total RNA

Proteins in experimental granuloma

Collagen

The amount of collagen in Ivalon sponge granulomas in rats begins to increase 7 days after the implantation and reaches a maximum on the 35th day (Boucek and Noble 1955 Woessner and Boucek 1961) Boucek and Noble (1957) found that in female rats the amount of collagen per gram of sponge rose to a maximum during 21 days and then remained constant for 300 days while in male rats the amount of collagen per gram of sponge rose from the 7th day to the 300th day When viscose cellulose implants were studied (Viljanto and Kulonen 196 Viljanto 1964) the amount of collagen per piece of sponge started to increase on the 5th day and continued to increase during the subsequent 55 days The amount of neutral salt soluble collagen (NSC) expressed as micrograms of protein bound hydroxyproline per 75 mg of sponge remained almost constant during the first 5 days but then rose to a maximum on the 21st day The amount of citrate soluble collagen (ASC) remained constant but the amount of insoluble collagen (ISC) rose rapidly after the first week of granuloma development Statistical analyses suggested that ISC is derived from NSC, but that ASC is a degradation product of ISC The tensile strength of the granuloma was first mainly due to NSC and later to ISC

Smirnov *et al* (1964) studied the incorporation of C proline into collagen in implanted Ivalon sponges as a function of age The specific activity of the collagen was a maximum on the 8th day but then decreased and rose to a second maximum on the 28th day Lampiaho and Kulonen (196) found the incorporation of C proline as hydroxyproline into collagen in viscose cellulose granulomas *in vitro* to be maximal from the 10th to 30th day The capacity to produce collagen was maximal during a surprisingly short period the rate of synthesis increased abruptly on about the 10th day and was a maximum on the 21st day The amount of C proline incorporated as hydroxyproline in collagen on the 43rd day was only one fifth of that incorporated on the 21st day The synthesis of collagen depended on the supply of oxygen and glucose The formation of lactic acid by granuloma tissue *in vitro* correlated with the production of collagen. Ahonen and Kulonen (1966b) observed maximal incorporation of C glycine into collagen in 14 and 21 day old granulomas *in vitro*

The mechanism of collagen synthesis

Collagen differs from other proteins in primary structure It contains two specific amino acid hydroxyproline and hydroxylysine and is very rich in glycine (one third) and proline (Grassmann *et al* 1965) The basic unit in collagen fibres is tropocollagen, which consists of three polypeptide chains (Piez 1961 1963) of which two are almost identical ($\alpha 1$ and $\alpha 3$) and one different ($\alpha 2$) in amino acid composition

The biosynthesis of collagen can be divided into three phases (Grassmann 1965) (1) the formation of the polypeptide chain (2) the hydroxylation of proline and lysine (3) and the formation of collagen fibres

declined The incorporation of radioactive phosphate into DNA was rapid during the first five days although the phosphate pool remained constant throughout the period of tudy (5—17 days)

Ribonucleic acids

Viljanto (1964) and Viljanto and Kulonen (1962) studied the variation of the content of RNA in visco e cellulose granulomas with age RNA was extracted together with DNA by the method of Biggers *et al* (1961) and determined by measuring the UV absorption The amount of RNA increa ed during the ob ervation period (1—60 days) it was about 2 1 mg/100 mg of sponge on the 4th day and 3 3 mg/100 mg of sponge on the 60th day The maximum amount of RNA recorded by Lampiaho and Kulonen (196) was 5 2 mg/100 mg of vi cose cellulose, this value was reached during the fourth week of development The amount decreased from the 40th to the 90th day

The incorporation of C uracil *in vivo* into RNA in polyvinyl sponge granulomas was studied by Bashey, Woessner and Boucek (1964) They i olated RNA with phenol after homogenizing the tissue in 0 9 % odium chloride solution The RNA content was calculated from the content of purine bound ribose (Brown 1946) The amount of RNA was about 0 7 mg/100 mg of sponge on the 8th day 0 9 mg/100 mg on the 13th day and 1 5 mg/100 mg on the 20th day Le s RNA per 100 mg of ponge was produced in female than in male rats These amounts of RNA are lower than the amounts reported by Viljanto (1964) Ba hey, Woessner and Boucek claimed that aqueous phenol extracts only oluble RNA The specific activity of RNA labelled by C uracil injected 6 hours before sacrifice increa ed linearly with tissue age

The incorporation of ^{32}P phosphate into RNA in implanted polyvinyl sponges was studied by Smirnov *et al* (1964) RNA was extracted with a 0 5 % sodium dodecyl sulphate solution and 90 % aqueous phenol The specific activity of RNA was the same on the 8th and 15th days and a maximum on the 29th day It was found that all RNA fractions resolved by density gradient analysis and methylated albumin chromatography were labelled 24 hours after the injection of ^{32}I phosphate

Ribonucleoprotein particles (ribosomes) in experimental granulomas were studied by Malt and Speakman (1964) and Kumento *et al* (1965) The former investigators tried to isolate polysomes involved in collagen synthesis by density gradient resolution of the post mitochondrial upernatant obtained after incubating granuloma slices with ^{14}C proline They were able to confirm the result obtained by Kret inger *et al* (1964) with chick embryos that collagen synthesis probably occurs in polysomes that have sedimentation coefficients over 350S Kumento *et al* tudied ribo omes isolated from granulomas 8 19 and 60 days old by ultracentrifugation but did not find any definite differen es Th y found bound H hydroxyproline in nuclear and cytoplasmic fractions i olated after incubating granuloma lices with ^{3}H proline

Williamson and Guschlbauer (1961 1963) studied RNA in regenerating wounds The amount of RNA per mg of tissue nitrogen determined by Schmidt Thannhau er extraction (Schmidt and Thannhauser 1945) and the phosphorus content ro e from the 5th to the 9th day and then decreased to the 17th day The ratio of purines to pyrimidines determined by phosphorus and purine bound ribose analyses decreased from 1 11 on the 5th day to 0 61 on the 17th day The incorporation of ^{32}I phosphate reached a maximum 24 hours after its introduction The specific activity of RNA d crea ed with increasing age of the wound The authors studied also the distribution of RNA in

ubcellular fractions obtained by differential centrifugation of wound tissue homo genates The nuclear fraction contained 50 % the micro omal fraction 10 % and the nonparticulate fraction 45 % of the total PNA

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Th bio ynth e is of collagen can be divided into three phases (Gra smann 1965) (1) the formation of the polypeptide chain (2) the hydroxylation of proline and lysin (3) and the formation of collag n fibres

Gerber and Altman (1961) suggested that the biosynthesis of collagen differs from that of other proteins. They proposed that preformed polypeptide blocks add to pre-existing collagen fibres. Their argument was based on the fact that a collagen molecule is considerably larger than the ribosome, which was then thought to be the template in protein synthesis. The polysome concept (Wettstein *et al.* 1963), however, allows the biosynthesis of very large molecules in ribosomes. More recent data suggest that collagen is synthesized in microsomes (Lowther, Green and Chapman 1961, Eastoe 1961, Prockop, Peterkofsky and Udenfriend 1962) and that the synthesis is directed by an mRNA. Bekhor and Bavetta (1965) found that actinomycin D inhibits collagen synthesis in rat skin slices *in vitro*. The effect of actinomycin D on collagen synthesis was studied also by Jeffrey and Martin (1966), who found that after 12 hours' incubation of chick embryo tibias with the antibiotic, the incorporation of ^{14}C proline into protein-bound hydroxyproline ceased. However, Lampiaho and Kulonen (1967) found that actinomycin D has only a weak effect on the synthesis of collagen in granuloma slices *in vitro*.

The second phase in collagen biosynthesis is the hydroxylation of the nascent polypeptide chain (Udenfriend 1966). It has been shown that free hydroxyproline is not a precursor of collagen-bound hydroxyproline (Stetten 1949, Green and Lowther 1959), but that incorporated proline is hydroxylated to hydroxyproline. Oxygen required in the hydroxylation reaction is derived from molecular oxygen (Fujimoto and Tamiya 1962, Prockop *et al.* 1962). The substrate of the hydroxylase is a proline-rich polypeptide. Peterkofsky and Udenfriend (1963) found a lag period in the incorporation of ^{14}C proline into collagen hydroxyproline in a cell-free system synthesizing collagen, but ^{14}C proline was incorporated into protein right from the beginning of the experiment. Oxygen was necessary for the former process but not for the latter. RNAse inhibited ^{14}C proline incorporation but not the formation of ^{14}C hydroxyproline from ^{14}C proline. Later it was shown that a hydroxyproline-deficient protein hydrolysable by purified collagenase accumulates in collagen-synthesizing systems when no oxygen is available. This proline-rich polypeptide is hydroxylated in the presence of oxygen (Peterkofsky and Udenfriend 1965, Prockop and Juva 1965). It was also shown that synthetic poly (L-prolyl-glycyl-L-prolyl) is hydroxylated by chick embryo homogenates (Juva and Prockop 1965).

Although biosynthesis of collagen has not been demonstrated to occur in a cell-free system consisting of purified ribosomes, tRNA, mRNA and energy, the data on the biosynthesis of collagen suggest that the polypeptide chain of collagen is synthesized by a similar mechanism as other proteins. The hydroxylation of proline takes place in the nascent polypeptide chain (Juva 1968).

Non-collagenous proteins

Kao, Boucek and Noble (1957) studied the amount and amino acid composition of sodium chloride-soluble proteins in Ivalon granulomas. The content of soluble protein per gram of Ivalon sponge was constant during the development of the granuloma. This was confirmed by Woessner and Boucek (1961a). Bollet *et al.* (1958) found that the amount of protein per gram of tissue was constant during the first three weeks and then decreased. Kao, Boucek and Noble (1957) and Viljanto (1964) studied the electrophoretic distribution of soluble proteins from experimental granulomas. The pattern resembled that of serum proteins during the first two days

Lampiaho and Kulonen (1967) followed the incorporation of ^{14}C proline into non gelatinized protein from granulomas The rate of incorporation was maximal from the 10th to 29th day but 30 % to 50 % lower during the 40th to 74th days

Woessner and Boucek (1961b) measured the activities of several enzymes in Ivalon granulomas as a function of age The activities were compared with the amounts of DNA and collagen They concluded that glutamic oxalacetic transaminase imidopeptidase iminopeptidase cytochrome reductase catalase and phenol sulphatase are present in constant amounts in the fibroblasts at all stages of development and are indirectly involved in collagen synthesis whereas alkaline phosphatase peroxidase and lactic and malic dehydrogenases are directly involved in proliferation but not in collagen synthesis Proteinase (pH optimum at pH 3 2) β glucuronidase and acid phosphatase are related to fibroblast differentiation and may be involved in collagen degradation

PURPOSES OF THE PRESENT INVESTIGATION

The purposes of the present investigation were

(1) to develop methods for the extraction and fractionation of nucleic acids from experimental granulation tissue

(2) to study the biosynthesis of nucleic acids and proteins in granulation tissue in different phases of development,

(3) to study whether the various phases of granulation tissue development (cell proliferation differentiation for collagen synthesis, involution) are associated with qualitative changes in the nucleic acids

METHODS

Biological experiments

Production of granulomas

Granulomas were produced as described by Viljanto and Kulonen (1962) and Viljanto (1964) by subcutaneous implantation of viscose cellulose sponges in male rats of a white Wistar strain At the time of implantation the rats were about 2 months old and weighed 150—180 g The animals were kept on a standard laboratory diet consisting of soy bean maize mill powder margarine sodium chloride and calcium carbonate Once a week they were given fresh vegetables cod liver yeast and fresh lung of cattle Water was given *ad libitum* The animals were housed in stainless steel cages at 20—25°

The implantation material was viscose cellulose sponge (Visella brand Säteri Oy Valkeakoski Finland) in the form of pieces 10×10×20 mm weighing 80—85 mg The pieces and the instruments were sterilized in 0 9 % sodium chloride solution at 100° for 30 min The rats were anaesthetized with ether and the dorsal skins were shaved and rinsed with ethanol An incision was made in the dorsal midline and four sponges were placed under the skin The wound was closed with a continuous silk suture After the operation the animals were placed in individual cages and kept on the standard diet

Incorporation experiments

Incorporation of ^{32}P phosphate in vivo A dose of 0 8—10 mCi of phosphate ^{32}P (Sodium Phosphate ^{32}P Injection PBS 2P The Radiochemical Centre Amersham Bucks England) in 1—2 ml of 0 9 % sodium chloride solution was injected *intraperitoneally* into sponge bearing rats under ether anaesthesia After periods varying from ½ to 12 hrs the sponges were removed under ether anaesthesia and cooled on crushed ice after which the remnants of the capsule were removed and the tissue containing sponges were homogenized

Incorporation of ^{14}C glycine in vitro Granulomas were removed by sharp dissection under ether anaesthesia and immersed in cold incubation medium They were then freed of remnants of the capsule and sliced with a Stadie Riggs microtome in a cold room A sample was taken for dry weight determination

The incubation was carried out in air in a metabolic shaker (Compenstat, Gallenkamp & Co, London England) at +38° The medium was the Krebs Ringer phosphate medium, pH 7 4, (Umbreit 1957) supplemented with amino acids (L leucine 0 76 mM L isoleucine 0 38 mM, L arginine 0 95 mM L-lysine 1 10 mM L tyrosine 0 22 mM L glutamine 0 85 mM, valine 0 85 mM L threonine 0 84 mM, L methionine 0 13 mM and glucose 22 4 mM) as described by Green and Lowther (1959) Penicillin (Na Penicillin Leiras Turku Finland 75000 units/litre) and streptomycin (Streptocin Laake Oy, Turku Finland 6 mg/litre) were added to each flask Thirty millilitres of the medium and slices from 4 granulomas (wet weight about 4 g) were added to each flask After the contents of the flask had warmed to 38° radioactive glycine (Glycine ^{14}C CFB 11, The Radiochemical Centre) was added 12 5 μCi per flask The incubation was continued for 4 hours with vigorous shaking of the sample mixture

The incubation was stopped by chilling the flasks in an acetone dry ice mixture The slices were separated from the medium by centrifuging in a refrigerated centrifuge (MSE High Speed 17, Measuring and Scientific Equipment Ltd London England) at 10 000 *g* for 10 minutes at 0° The slices were washed on a Buchner funnel with cold incubation medium which contained non radioactive glycine and dried between filter papers

Incorporation of ^{3}H cytidine and ^{3}H uridine in vitro The experiment was performed as described above, except that either ^{3}H cytidine (TRA 198 The Radiochemical Centre) or ^{3}H uridine (TRA 27 The Radiochemical Centre) was added instead of ^{14}C glycine Fifty millilitres of incubation medium and 200 μCi of labelled nucleosides were added to the slices from 8 granulomas (wet weight 7—8 g)

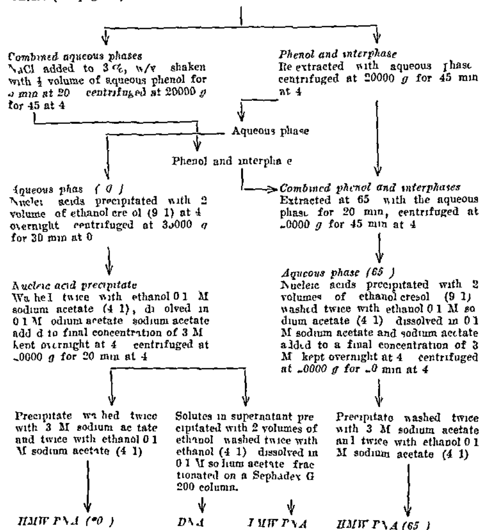
Extraction and fractionation of nucleic acids and proteins from granulomas

General extraction method

The extraction and fractionation procedure was that proposed by Kirby (1965) except that sodium deoxycholate (DOCA) or sodium dodecyl sulphate was present in the aqueous phase of the phenolic extraction mixture (Fig 1)

Fig 1 Extraction and fractionation of nucleic acids from granulomas

Granuloma tissue homogenized in 10—15 volumes (v/w) of the phenolic extraction mixture (see page 15) shaken 20 min at 20 centrifuged at 20000 *g* for 45 min at 4



The tissue in 4 granulomas was homogenized in a revolving blade homogenizer (Edmund Buhler Tubingen Germany) at 50 000 rpm and 0° for 2 min in an extraction mixture which consisted of equal volumes (30 ml) of two phases (1) an *aqueous phase* containing 0 5 grams of sodium deoxycholate (E Merck AG, Darmstadt Germany) and 0 5 grams of sodium naphthalene 1 5 disulphonate (Fluka AG Basle Switzerland) in 100 ml of distilled water (2) a *phenolic phase* containing 100 ml of freshly

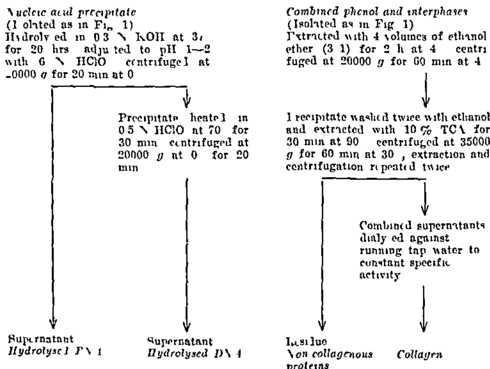
aqueous phenol (guaranteed reagent E. Merck AG), 14 ml of freshly distilled *m*-cresol (The British Drug House, Poole, England) and 0.1 gram of 8-hydroxyquinoline (for chromatography, E. Merck AG). Thirty millilitres of each phase was used for 4 granulomas (wet weight 4 g). A second extraction was performed at 65° to collect so-called nucleolo-chromosomal RNA from the interphase (Georgiev *et al.* 1963). The detailed extraction procedure is presented in Fig. 1.

When proteins and nucleic acids were extracted from granulomas (experiments with ^{14}C-glycine), the extraction at 65° was omitted. RNA were separated from DNA by alkaline hydrolysis. Proteins were precipitated from the interphase and collagen was extracted by the method of Fitch, Harkness and Harkness (1955) (Fig. 2).

Fractionation of the nucleic acid preparation

High molecular weight RNA (HMW RNA) were precipitated from the nucleic acid solution with 3 M sodium acetate (see Fig. 1). DNA remaining in the supernatant were separated from LMW RNA by gel filtration (Ahonen and Kulonen 1966a; Dehlins and Staehelin 1966).

Fig. 2. Extraction of nucleic acids and proteins from incubated granuloma slices



Extraction of total RNA

A modification of the original Schmidt—Thannhauser procedure (Schmidt—Thannhauser 1945) was used The granulomas were homogenized in cold 0 6 N perchloric acid solution 10 ml/g of tissue (wet weight) and centrifuged at 35 000 *g* and 0° for 60 min The precipitate was washed twice with cold 0 6 N perchloric acid solution and once with 80 % (v/v) ethanol The precipitate which contained RNA and DNA was incubated in 0 3 N potassium hydroxide solution at 37° for 20 hrs Hydrolysed RNA were separated from DNA and proteins by precipitating the latter by adjusting the pH to 1—2 with perchloric acid and centrifuging The supernatant was used for the determination of the nucleotide composition of the RNA

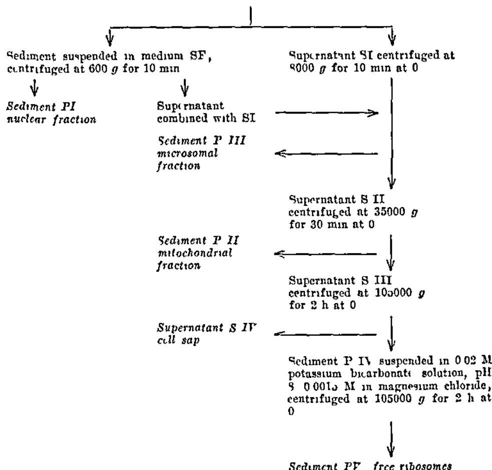
Subcellular fractionation of granulation tissue

The granulomas were homogenized with the revolving blade homogenizer at 50 000 rpm and 0° for 30 seconds in medium SF pH 6 8 which was 0 32 M in sucrose 0 001 M in KH_2PO_4 and 0 001 M in $MgCl_2$ One hundred millilitres of the medium SF was used for every ten implants Before homogenization 0 1 g of magnesium bentonite was added to every 10 granulomas (Petermann 1964) The fractionation procedure is presented in Fig 3

Sediment PI which contains nuclei collagen fibres and cells according to Viljanto (1964), was used as such for the isolation of nuclear sap and nucleolo-chromosomal RNA For the determination of the nucleotide composition of nuclear RNA nuclei were recovered according to Dingman and Sporn (1964) the pellet was suspended in 4 5 ml of the medium SF and hypertonic sucrose solution (2 39 M in sucrose 0 001 M in $MgCl_2$ 0 0035 M in K_2HPO_4 0 0007 M in ATP pH 6 9) was added to give a final volume of 33 0 ml After mixing the suspension was spun for 2 hrs at 50 000 *g* and 4° in a preparative rotor A of a Spinco Model E centrifuge (Beckman Instruments Inc Fullerton Calif U S A) to isolate the nuclear fraction as a gelatinous pellet

Sediment PII contains mitochondria and large microsomes Purification was attempted by centrifugation in sucrose density gradients 30—65 % sucrose in medium SF (see page 23) For the determination of the nucleotide composition of RNA PII was used without purification

When both free and microsomal ribosomes were isolated the 8 000 *g* supernatant was adjusted in an ice bath to pH 8 with 0 1 N sodium hydroxide solution Fresh 5 % sodium deoxycholate solution was added drop



wise with constant stirring to a final deoxycholate concentration of 0 5 % The solution was centrifuged at 105 000 *g* at 2° for 2 hrs in a Spinco Model L Ultracentrifuge, rotor No 40 (courtesy of Professor Hartiala, Department of Physiology, University of Turku) The sediment containing the ribosomes was suspended in cold 0 02 M potassium bicarbonate solution of pH 8 0 0 0015 M in magnesium chloride and recentrifuged at 105 000 *g*

Cell sap RNA were isolated from supernatant SIV by precipitation with two volumes of ethanol and subjected to alkaline hydrolysis

For the isolation of the subribosomal particles, the 8 000 *g* supernatant (SII) [illegible] ted in a zonal rotor (BXV, MSI Super speed 65 ultra centrifu[illegible] [illegible]aration was carried out at 25 000 rpm for 9 h at

about 4° in a sucrose density gradient 10—47 % in Medium SF The particles were sedimented from the fractions by adding two volumes of ethanol and cooling to —5°

For the determination of the nucleotide compositions RNA from the subcellular fractions was isolated by alkaline hydrolysis The DOCA phenol method was used to isolate RNA for macromolecular characterization

Sediment PI contained 93 5 % of the total DNA of the tissue The subcellular fractions were also examined in an electron microscope PI contained besides nuclei, unbroken cells collagen fibres and sponge debris PII consisted of mitochondria and some microsomal structures

Chemical analysis of the nucleic acid fractions

Determination of the base composition of DNA

The DNA sample was weighed in a test tube and 0 5 ml of formic acid (98—100 %, E Merck AG) was added per milligram of DNA The tube was flushed with nitrogen and sealed by fusion The hydrolysis was performed at 175° for 2 h The solvent was then evaporated under reduced pressure and the residue dissolved in 100μl of N hydrochloric acid solution (Fischer and Chargaft 1948)

The purine and pyrimidine bases were separated by chromatography on Whatman No 1 paper (H Reeve Angel & Co Ltd , London, England) using 2 propanol hydrochloric acid water (17 4 4) as solvent(Wyatt 1951) For identification, *n* butanol ammonia water (20 1 3) was used in the second dimension The spots were located with UV light of 2540 Å (Universal UV lampe TL 900 Camag Muttenz Switzerland) cut out and eluted with 0 1 N hydrochloric acid The eluates were filtered and their absorbances were measured at 2600 Å in a Beckman DU spectro photometer The amounts of the individual bases were calculated using the following millimolar extinction coefficients cytosine 6 0 adenine 13 0 thymine 7 4 guanine 7 2 (Beaven Holiday and Johnson 1955) Commercial purine and pyrimidine bases (L Light & Co Ltd Colnbrook Bucks England) were used as reference compounds

Determination of the nucleotide composition of RNA

Separation of nucleotides by chromatography on anion exchange columns The RNA nucleotide solution was adjusted to pH 9—10 and allowed to drain into a Dowex 1 column (1 × 10 cm) The resin (Dowex 1 × 8 100—200 mesh Fluka AG Buchs Switzerland) was pretreated

according to Morell Ayers and Greenwalt (1962) The column was first eluted with 20 ml of water and then with two acid gradients (1) 100 ml water in the mixing vessel (closed cylinder) and 0 2 N formic acid (pH 2 1) in the reservoir until AMP 3 emerged (2) 0 2 N formic acid (pH 2 1) in the mixer and 0 5 M ammonium formate in 0 1 N formic acid (pH 4 2) in the reservoir The flow was adjusted with a Desaga peristaltic pump to 1 ml/min and 10 ml fractions were collected The absorption of the eluates was followed at 2540 Å with a Uvicord ultraviolet absorptiometer (LKB Produkter Ab, Stockholm Sweden) connected to a recorder (Single Point Model DIE1 Cambridge Instrument Company Ltd London, England) that was set to give a full scale deflection of 10 mV

The fractions were pooled and the UV absorption was measured at 2500 Å 2600 Å 2800 Å and 2900 Å The amount of each nucleotide was calculated using the following millimolar extinction coefficients at 2600 Å CMP 6 8 AMP 14 2 UMP 10 0 GMP 11 8 (Beaven Holiday and Johnson 1955)

The nucleotides were identified on the basis of their positions on the chromatogram their spectral properties their R_f values in chromatography on Whatman No 1 paper (*isobutyric* acid ammonia water, 4 0 0002 1) and their migration in electrophoresis on cellulose acetate sheets The reference compounds were obtained from the California Biochemical Corporation Los Angeles Calif U S A

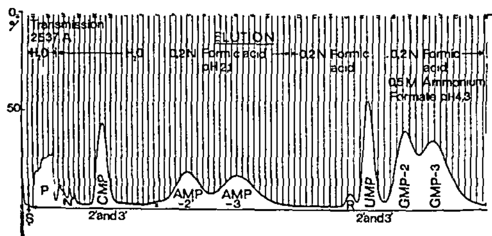


Fig 4 ... *chromatography of RNA hydrolysate* Alkaline hydrolysate of RNA from 90 h granulomas containing 11 mg of RNA applied to a 1 × 10 cm column of Dowex 1 8 100– 00 mesh S = point of application of the sample I = ... probably peptide eluted with water N = nucleotides R = 5 ribo ... phosphate (? UIM) The vertical lines were drawn by the event marker

A typical chromatogram is seen in Fig. 4. In order to determine the accuracy of the procedure six runs were performed with an artificial nucleotide mixture. The relative standard deviation of the mean mole percentage was 1.5 % for CMP, 3.5 % for AMP, 2.9 % for UMP and 1.8 % for GMP.

Separation of nucleotides by cation and anion exchange chromatography. The RNA sample (50—100 μg) was hydrolysed in 0.3 N potassium hydroxide at 37° for 20 hrs. After neutralization with perchloric acid, the nucleotide mixture was made 0.05 N in hydrochloric acid and absorbed on a 0.8 cm × 2 cm column of Dowex 50W × 8 resin (100—200 mesh, J. T. Baker Chemical Co., Phillipsburg, N. J., U.S.A.) in the H^+ form prepared as described by Smith (1960) and equilibrated with 0.05 N hydrochloric acid. The eluate was monitored as before. The column was eluted with 0.05 N hydrochloric acid until the first peak emerged from the column and the elution was then continued with distilled water. The fractions were collected manually.

The amounts of the individual nucleotides were computed from their UV absorbance values as described by Katz and Comb (1963).

When it was necessary to separate CMP from AMP, the eluate remaining after GMP had been removed was absorbed directly on a 0.8 cm × 2 cm column of Dowex 1 prepared as described above. The column was eluted first with 0.05 N formic acid to remove CMP and then with 0.4 N formic acid to remove AMP.

The elution profile is seen in Fig. 5. To test the reproducibility of the method, a sample of RNA isolated from rat liver was hydrolysed in 0.3 N potassium hydroxide and six determinations of the nucleotide composition were carried out. It was found that the relative standard deviation of the mole percentage was 4.9 % for CMP, 4.5 % for AMP, 1.0 % for UMP and 3.2 % for GMP.

Analysis of the nucleotide composition of RNA by means of ^{32}P *phosphate.* RNA labelled with radioactive phosphate were hydrolysed in 0.3 N potassium hydroxide solution together with nonradioactive carrier RNA. The liberated mononucleotides were separated by combined anion and cation exchange chromatography and the radioactivities were measured and corrected for quenching using a ^{32}P phosphate standard.

When five HMW RNA samples from 14-day granulomas (^{32}P phosphate incorporated for 2 h) from different animals were analysed, it was found that the relative standard deviation of the mole percentage was 6.1 % for CMP, 10.0 % for AMP, 5.2 % for UMP and 6.6 % for GMP. At least 3000 cpm above the background were recorded in each case.

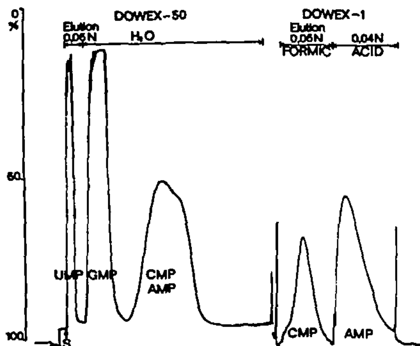


Fig 5 Combined cation and anion exchange chromatography of RNA hydrolysate Alkaline hydrolysate of HMW RNA (0 12 mg) extracted at 20 from 14 day granulomas applied to a 0 8 × 2 cm column of Dowex 50 × 8 100—200 mesh S = point of application of the sample UMP was eluted with 0 05 N HCl GMP with water, both were collected manually CMP and AMP emerged together after GMP When the elution of AMP + CMP from Dowex 50 was complete as indicated by the recorder, the Dowex 1 × 8 (100—200 mesh) column 0 8 × 2 cm was connected to the monitoring system and CMP was first eluted with 0 05 N formic acid and then AMP with 0 4 N formic acid

Correction of the mole percentages of nucleotides CMP was deaminated to UMP in alkaline solution (Marrian *et al* 1951) The correction coefficients used were

corrected amount of CMP = 1 10 × measured amount of CMP

corrected amount of UMP =

measured amount of UMP — 0 06 corrected amount of CMP

(Ahonen and Kulonen 1968)

Choice of the method Chromatography on an anion exchange column was found suitable for the isolation of RNA released from granulomas or subcellular fractions by direct alkaline hydrolysis since the contaminating peptides were eluted before nucleosides It was suitable also for the analysis of LMW RNA since ψ-UMP was separated from UMP The method can be used only with relatively large amounts of RNA, at least 1 mg being

needed, it is however, time consuming (about 14 h). Chromatography on a cation exchange resin is rapid (about 1 h) and suitable for analysis of 50—200 μg of RNA and so it was used for the analysis of DOCA phenol extracted RNA. Combined cation and anion exchange chromatography was found suitable for analysis of the distribution of ^{32}P among nucleotides of RNA.

Physical analysis of nucleic acids

Ultracentrifugation

Density gradient. An MSE Super Speed 50 S centrifuge equipped with a 3 × 23 ml swing out rotor and an electronic temperature controlling device was used. Runs were made either at 21 000 rpm giving a minimum centrifugal force of 32 000 *g* and a maximum force of 60 000 *g* or at 30 000 rpm giving forces of 69 000 *g* and 129 000 *g*, respectively. All the runs were carried out at 4°.

Linear sucrose gradients from 5 to 20 % in 0.1 or 0.01 M sodium acetate solution were produced according to Davis, Sinten and Agranoff (1965). A Desaga peristaltic pump equipped with the tubing set C (silicone rubber, internal diameter 0.5 mm) was used.

The RNA sample to be analysed was dissolved in 0.1 M sodium acetate (1—2 mg/ml) solution and 0.5—1 ml of the solution was carefully layered on top of each density gradient.

After the centrifugation the rotor was removed and the tube was carefully transferred to the gradient evaluator (Fig. 6). Silicone grease was applied to the bottom of the tube before it was placed in the tube holder. The bottom of the tube was punctured with a hypodermic needle which was connected to tubing from the peristaltic pump delivering 70 % sucrose solution. Both the tube holder and the sucrose solution were kept in an ice bath. The top of the tube holder was connected to the UV monitoring system described before. The flow rate was 0.25 ml/min.

If the radioactivity was to be determined, 0.025—0.5 ml fractions were collected in an automatic timer operated fraction collector (a model designed and made in our workshop). To each fraction 1 ml of 0.01 M sodium acetate solution was added followed by 1 ml of a mixture of *n*-butanol (100 ml), 2,2-diethyldihexylamine (11.7 ml) and glacial acetic acid (2 ml) (Kidson *et al.* 1963). After the mixture had been shaken and the phases had been allowed to separate, 0.5 ml of the organic phase was transferred to the counting vial.

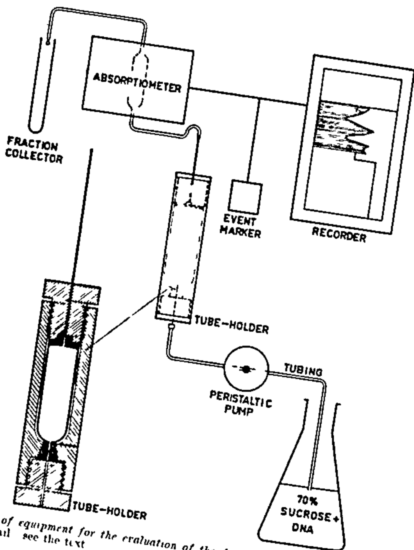


Fig 6 Scheme of equipment for the evaluation of the density gradient sedimentation pattern For detail see the text

The sedimentation coefficients were measured according to Martin and Ames (1961) The 16S fraction was used as the marker and the S values were calculated from the equation

$$S_x = \frac{\text{distance of X from the top of the gradient}}{\text{distance of the marker from the top of the gradient}} \times 16$$

Analytical ultracentrifugation A Spinco Model L analytical ultracentrifuge was used The schlieren optical system of the ultracentrifuge was equipped with a phase plate as the schlieren diaphragm The tem

perature during the runs was +19—+21° Sector shaped cells (12 mm high with a sector angle of 2°) were used The solvent was a solution of pH 5 1 0 1 M in sodium acetate and 0 025 M in sodium chloride in all runs, which all were performed with two concentrations of the solute

The sedimentation coefficients were determined as described by Schachman (1957) In order to obtain sedimentation coefficients at infinite dilution (S°_{ow}), the values of $1/S_{ow}$ were plotted against concentration

Chromatography on methylated serum albumin

The methylated albumin kieselguhr (MAK) columns were prepared according to Mandell and Hershey (1960) Bovine albumin powder (fraction V Armour & Co) and Hyflo Super Cel (L Light & Co) were used The nucleic acid to be analysed was dissolved in 25 ml of a 0 1 N solution of sodium chloride in 0 05 M phosphate buffer of pH 6 7 and passed through a column at a flow rate of 15 ml/h After the column had been washed with two 1 ml portions of 0 1 M sodium chloride the elution was started with a linear gradient of sodium chloride in 0 05 M phosphate solution of pH 6 7 produced in a Varigrad apparatus (prepared in our workshop to the design of Petersen and Rowland (1961))

Chromatography on DEAE Sephadex

Nucleic acids were also fractionated on DEAE Sephadex A 25 (coarse Pharmacia Ab) The anion exchange material was prepared for chromatography according to the manufacturer s instructions Columns with an internal diameter of 1 5 cm and a height of 25 cm were used A sodium chloride gradient in a 0 01 M phosphate buffer of pH 6 7 was used for elution (Fig 8)

Spectrophotometric analysis

A Beckman DU spectrophotometer the wave length setting of which was checked using a didymium filter was used

The hyperchromicities were determined by heating the samples in stoppered cuvettes to 90° After reading the absorbance at 2600 Å the solutions were left to stand at room temperature and their absorbances were measured again when the temperature of the solution had fallen to 20°

Formation of DNA RNA hybrids

The method of Armstrong and Boezi (1965) was used The DNA were extracted from 14 day granulomas and purified as described above They were dissolved (1 mg/ml) in 0 06 M KCl—0 01 M Tris buffer of pH 7 3 and denatured at 100° for 10 min (the hyperchromic effect was 30 3 %) ³P labelled RNA were dissolved (10μg/ml 1000—10 000 cpm/ml) in 0 5 M KCl—0 01 M Tris buffer of pH 7 3 DNA were added to give a final concentration of 100 μg/ml and the solution incubated at 78° for 2 h After incubation, the solution was cooled to 37° and 0 1—1 0 ml samples were added to 0 5—5 0 ml of 0 06 M KCl—0 01 M Tris buffer of pH 7 2 containing 10—100 μg of pancreatic RNAse (Fluka AG, crystallized four times) and incubated at 37° for 10 min Then 15 ml of 0 5 M KCl solution in 0 01 Tris buffer of pH 7 3 was added and the solution passed through a Millipore HA filter (pore diameter 0 45 μ Millipore Filter Corporation, Bedford, Mass U S A) The filter was washed with six 10 ml portions of the 0 5 M KCl solution in 0 01 M Tris buffer of pH 7 3, dried and its radio activity determined Control samples of RNA which contained no DNA were treated similarly to correct the results for any nonspecific absorption by the filter In order to determine the proportion of radioactive RNA that hybridized, control samples were precipitated with 10 % trichloro acetic acid on Millipore filters washed with 5 % trichloroacetic acid and 70 % ethanol and dried and their radioactivities determined The radio activities of the filters were determined by adding 5 ml of the scintillation liquid to the counting vials

Assay of amino acid acceptor capacity

Amino acid activating enzymes were isolated from 40 rat livers according to Zachau (1964) The enzyme preparation obtained by DLAI cellulose chromatography contained 14 mg of protein per ml It was dissolved in a 0 02 M potassium phosphate buffer of pH 7 7 0 25 M in sodium chloride and stored at —20°

The nucleic acid sample was incubated together with 0 05 μCi of ³H lysine (TRA 232 The Radiochemical Centre) in 0 8 ml of 25 μM Tris HCl buffer of pH 7 5 containing 1 mg of amino acid activating enzymes 10 μM ATP and 15 μM $MgCl_2$ for 15 min at 37° The incubation was terminated by adding 5 ml of cold 5 % trichloroacetic acid The precipitate was separated by centrifugation and washed thrice with cold 5 % trichloro

acetic acid and once with 80 % ethanol The precipitate was dissolved in dilute sodium hydroxide solution and the mixture was neutralized and added to a counting vial containing methyl cellosolve and scintillation liquid for the determination of radioactivity

Other analytical methods

DNA were determined by the diphenylamine method (Burton 1956) The standard was DNA obtained from granulomas by the DOCA phenol method and purified with cetyltrimethylammonium bromide (Jones 1963)

For the characterization of the sugar moieties in nucleic acid fractions the diphenylamine reaction was used for deoxyribose and trimethylsilylation and gas chromatography according to Karkkainen Lehtonen and Nikkari (1965) was used for ribose

Phosphorus was determined according to Fiske and Subbarow (Hawk Oser and Summerson 1954)

Hydroxyproline nitrogen and *protein* were determined as described by Pikkarainen (1968)

Measurement of radioactivity Radioactivities were measured by internal liquid scintillation counting using a Packard Tricarb Model 3024 Spectrometer (Packard Instruments Company Downers Grove Ill U S A) The solvent system (Prockop and Ebert 1960) consisted of 6 ml of ethylene glycol monomethyl ether (methyl cellosolve) (Shell) and 10 ml of scintillation liquid (15 g PPO (2 5 diphenyloxazole) and 50 mg POPOP ((4 methyl 5 phenyloxazolyl)benzene) (Packard) in 1000 ml of distilled toluene) This system dissolved 0 5 ml of an aqueous solution ^{14}C counting efficiencies were individually determined using an internal standard ^{14}C toluene (specific activity $4.21 \times 10^5 \pm 2.17$ dmp/ml Packard) ^{32}P counts were corrected for decay when necessary by measuring the radioactivity of a standard solution prepared from each lot of ^{32}P phosphate at the same time as the radioactivities of the sample solutions

RESULTS

Extraction of nucleic acids from granulomas

As mentioned, the aim of the experiments was to find suitable methods for the extraction of nucleic acids as undegraded macromolecules from granulomas. Fourteen day granulomas were used as test material since according to existing data (Viljanto and Kulonen 1962, Viljanto 1964) they possess the typical features of granulation tissue and since the nucleic acid content is high in granulomas of this age.

The extraction method. From available information (Allen 1962, Kirby 1964) it was obvious that extraction with a phenol water mixture was the method of choice. However, preliminary experiments with phenol water mixtures gave poor yields of both RNA and DNA and so it was necessary to use more complex solutions containing other reagents. Sodium deoxycholate was chosen since it is known that ribosomes occur in close proximity of the membranes of endoplasmatic reticulum in fibroblasts (Ross and Bendich 1963) and since it has been used successfully to extract DNA from Ehrlich ascites cells (Colter, Brown and Ellem 1962). Another additive, sodium naphthalene 2 sulphonate, has been reported to increase the yield of RNA and to reduce the contamination of nucleic acids by protein (Kirby 1962).

Sodium deoxycholate was compared with sodium dodecyl sulphate which has been used to extract nucleic acids from rat brain (Sporn and Dingman 1962) and liver (Hiatt 1962).

Comparison of the nucleic acids extracted from 14 day granulomas by DOC phenol and SDS phenol mixtures. Nucleic acids were extracted from 14 day granulomas using 0.5 % SDS or 0.5 % DOCA solutions and 90 % aqueous phenol as described in Fig. 1. 58.1 % of the nucleic acids in the preparation extracted by DOCA phenol were insoluble in 3 M sodium acetate, whereas the corresponding figure for the SDS phenol extracted preparation was 10.8 %. Addition of RNAse inhibitors, bentonite (1 mg/ml in the aqueous phase) and polyvinyl sulphate (1 µg/ml) in

creased the yield of HMW RNA Ribonuclease activity (measured by the method of Lgami Takahashi and Uchida 1964) was demonstrable (at pH 7 4) only in the SDS phenol extracted preparation In contrast a good yield of RNA insoluble in 3 M sodium acetate was obtained from rat liver using SDS phenol

Analysis of total nucleic acids extracted from 14 day granulomas by DOCA phenol The nucleic acids obtained by the deoxycholate phenol method from 14 day granulomas were studied by chemical and physico chemical methods The content of phosphorus was 9 4 % and that of nitrogen 15 1 % The preparation contained 31 8 % DNA (diphenylamine test) and 67 1 % RNA (calculated from the amounts of nucleotides liberated by alkaline hydrolysis) The protein content was less than 0 5 % No hydroxyproline was detected by the Stegemann Woessner test after hydrolysis of the preparation in 6 N hydrochloric acid

The sedimentation pattern of the nucleic acids in analytical ultra centrifugation is seen in Fig 7 Three main components ($s_{20,w}$ =9 0S 16 1S

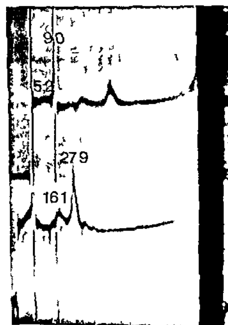


Fig 7 Schlieren diagrams of total nucleic acids extracted by the DOCA phenol method from 14 day granulomas in an analytical ultracentrifuge The picture was taken 13½ min after the rate of rotation rose to 59 780 rpm Temperature +19 0 during the run Upper curve 1 5 mg of nucleic acid per ml of 0 1 M sodium acetate—0 025 M sodium chloride pH 5 1 Lower curve 3 0 mg of nucleic acid per ml of the same buffer Sedimentation coefficients ($s_{20,w}$) are indicated in the figure

and 28.2S) and three minor components (s^{0}_{w} = 5.2S, 30S and 31S) were detected. The boundaries exhibited strong concentration dependence and hence the s_0 values were estimated using the two concentrations indicated in Fig. 7. The 9S material was isolated from the ultracentrifuge cells after the more rapidly moving components had sedimented to the bottom. It was identified as DNA on the basis of a positive diphenylamine test and the presence of thymine in the acid hydrolysate.

In order to measure the yield of nucleic acids, RNA and DNA were extracted from the residue by the Schmidt-Thannhauser procedure. It was found that 89 % of the total RNA and 54 % of the total DNA had been extracted by the DOCA-phenol mixture.

Fractionation of the nucleic acids

Chromatography of granuloma nucleic acids on DEAE Sephadex. Nucleic acids dissolved in 0.4 M NaCl—0.01 M phosphate were added to a column of DEAE Sephadex A 25 as described on page 25. Elution was performed with a sodium chloride concentration gradient, the pattern is seen in Fig. 8. Analytical results for the fractions are shown in Table 1. Addition of bentonite or polyvinyl sulphate did not affect the sedimen-

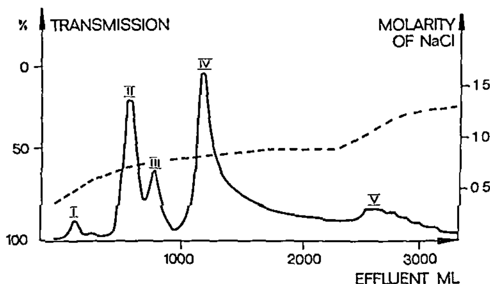


Fig. 8. Chromatography of total nucleic acids on DEAE Sephadex A 25. 27.1 mg of nucleic acids extracted from 14 day granulomas by the DOCA-phenol method was applied to the column. Analytical data for the fractions are presented in Table 2.

Table 1 Analysis of nucleic acid fractions obtained by DEAE Sephadex chromatography

Fraction (see Fig 4)	Sugar moiety	$s_{20,w}$	Nucleotide composition (mole %)	Amount of nucleic acids (mg)
I	Ribose	—	—	0 2
II	Ribose	2 1	CMP 31 2 AMP 18 0 UMP 19 8 GMP 31 0	2.2
III	Ribose	4 5	CMP 29 3 AMP 19 6 UMP 19 4 GMP 31 8	1 1
IV	Deoxy ribose	9 0	No alkali liberated nucleotides	4 2
V	Deoxy ribose	7 0	No alkali liberated nucleotides	0 5

97 1 mg of nucleic acid preparation obtained by DOCA phenol extraction from 14 day granulomas was adsorbed on the column 8 2 mg was recovered The nucleic acids were precipitated from solution with 75 % ethanol at —20 , washed free of sodium chloride with 75 % ethanol, and eluted through a Sephadex G 25 column with 0 1 M sodium acetate at 20

tation values or the recovery from the column Similar results were also obtained for rat liver RNA

DEAE Sephadex is not suitable for the fractionation of nucleic acids since HMW RNA cannot be eluted from the column

Salting out of granuloma nucleic acids Kirby (1965) has described a procedure for the separation of rRNA and rapidly labelled RNA (HMW RNA) from DNA and LMW RNA which involves extraction with 3 M sodium acetate DNA and LMW RNA are soluble in concentrated salt solutions while HMW RNA are not In experiments with granuloma nucleic

acids, some DNA always remained undissolved when the nucleic acids were extracted with 3 M sodium acetate However, when the nucleic acids were first dissolved in 0 1 M sodium acetate and sodium acetate was then added to a final concentration of 3 M HMW RNA precipitated, and neither DNA nor tRNA were detected in the precipitate (no thymine was found after paper chromatography of the acid hydrolysate nor was any 5 ribosyl uracil monophosphate detected by chromatography on an anion exchange column after alkaline hydrolysis of the precipitate) DNA and LMW RNA were separated by filtration through Sephadex G 200 gel as described in the methods section (Ahonen and Kulonen 1966a)

Characterization of the nucleic acid fractions

The nucleic acid fractions were characterized by various chemical and physicochemical methods (Table 2) Measured amino acid acceptor capacities are shown in Table 3

The '$s^0_{0\,w}$' value for the DNA was 9 0 A preparation with the same value was obtained also on using more gentle homogenization (pressing frozen tissue through an aperture 0 6 mm wide) The true $s^0_{0\,w}$ value could not be determined by the schlieren optical system, since the concentration dependence of the sedimentation constant is non linear at the concentrations that could be used (Oth 1955 Shooter and Butler 1956) The observed sedimentation rates agree well with those given by these authors In chromatography on MAK columns only one fraction was observed

The LMW RNA fraction contained transfer RNA as judged from the capacity to bind ^3H lysine in the presence of amino acid activating enzymes and ATP and from the presence of 5 ribosyluracil 2′(3′) monophosphate (ψ UMP) in the alkaline hydrolysate Analytical ultracentrifugation revealed only one peak $s^0_{20\,w}$ 4 6S but three distinct fractions were observed on MAK chromatography (Fig 9) The nucleosides present in the alkaline hydrolysate of LMW RNA were adenosine (0 3 mole %) and guanosine (0 6 mole %)

Two major peaks at 16 1S and 27 9S and a minor peak at approximately 23S were observed in the analytical ultracentrifugation of the HMW RNA fraction Only one peak was usually present in MAK chromatograms although partial separation into two fractions was observed occasionally The density gradient pattern of HMW RNA showed the presence of two main components The faster component corresponds to the 28S component in the analytical ultracentrifugation the slower component to the 16S component When centrifugation was continued at a

Table 2 Analysis of nucleic acid fractions extracted by the DOC-phenol from 14 day granulomas

Fraction	Sugar moiety	$s^{\circ}_{20,w}$	Hyperchromicity after heating	Hyperchromicity after recooling	Nucleotide composition (mole %) alkaline hydrolysate	Base composition (mole %) acid hydrolysate
DNA	Deoxyribose	9.0	30.7 %	13.5 %	—	C 21.8 A 27.5 T 27.4 G 22.1
HMW RNA	Ribose	16.1 23.0 [illegible]9	11.8 %	0.4 %	CMP 31.6 AMP 18.2 UMP 17.[illegible] GMP 32.6	—
LMW RNA	Ribose	4.6	[illegible]3.1 %	13.0 %	CMP 31.7 AMP 14.7 UMP 16.7 GMP 35.0 ψ UMP 1.8	—

Deoxyribose was detected by the diphenylamine reaction (Burton 1956), ribose by gas chromatography after hydrolysis in 1 N HCl and trimethylsilylation (Karkkainen, Lehtonen and Nikkari, 1965). The sedimentation coefficients were determined by analytical ultracentrifugation as described on page [illegible]4. Hyperchromicities were determined by heating the nucleic acid solution to 90° and slowly cooling to 20°; absorbances were measured at 2600 Å. Alkaline hydrolysis was performed in 0.3 N KOH at 37° for 20 h. The nucleotide composition of HMW RNA was determined by cation exchange chromatography (mean of 6 determinations), that of LMW RNA by chromatography on an anion exchange column (mean of 3 determinations). Acid hydrolysis was carried out in 98—100 % formic acid at 175° for 2 h and purine and pyrimidine bases were separated by paper chromatography.

higher speed for a longer time additional components were detected (Fig. 10). The sedimentation coefficients of these calculated according to Martin and Ames (1961) were 5S, 12S and 22S. The combined 5S, 12S and 22S fractions from several runs were dialysed several times against distilled water at +4°, passed through Sephadex C 25 in a 0.01 M sodium acetate buffer of pH 5.2 and lyophilized. The fractions were dissolved in water, eluted through a Sephadex G 25 column with 0.1 M sodium acetate solution and rerun in a 5—20 % sucrose gradient. The patterns of the 16S and

Table 3 Amino acid acceptor capacities of nucleic acid fractions extracted by the DOCA phenol method from 14 day granulomas

Enzyme preparation mg	Reaction mixture			Radioactivity (cpm) precipitated with TCA after incubation
	ATP μM	Buffer ml	Nucleic acid fraction, μg	
1.0	10	0.4	LMW RNA 30	16 400
0.5	10	0.5	LMW RNA 30	11 800
1.0	10	0.4	LMW RNA 2	2 260
1.0	10	0.4	LMW RNA 1	580
1.0	10	0.4	HMW RNA 150	240
1.0	10	0.4	DNA 130	1 170
0	10	0.6	LMW RNA 28	190
1.0	10	0.5	—	340
1.0	—	0.5	LMW RNA 30	520

The experiment was performed as described on page 26 5μCi of H lysine was added to each reaction mixture The results are means of three determinations

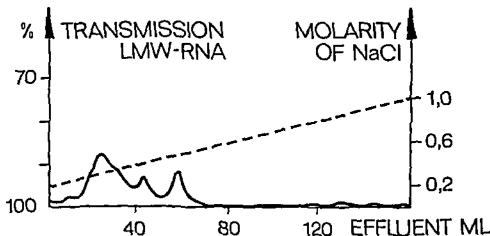


Fig 9 Chromatography of LMW RNA 1 from 14 day granulomas on a methylated albumin kieselguhr column The sample (0.4 mg) was dissolved in 0.1 M NaCl 0.05 M phosphate buffer pH 6.7 Elution was performed with a linear concentration gradient of NaCl from 0.2 M to 1.0 M in the phosphate buffer The eluting concentrations of NaCl are first peak 0.34 M second peak 0.40 M and third peak, 0.48 M

22S fractions are seen in Fig 10 The 5S and 12S fractions gave more complex patterns in similar runs It was estimated that the fraction 28S represented 65 % of the total HMW RNA the fraction 16S about 30 % the fraction 22S 3 % and the fractions 5S and 12S together about 3 %

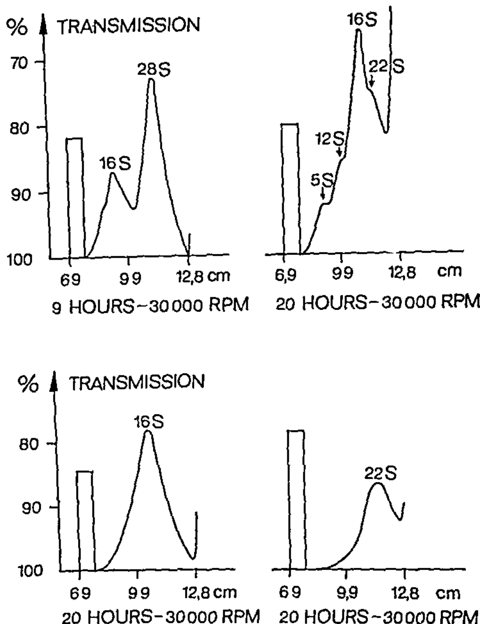


Fig 10 Sucrose density gradient analysis of HMW RNA from 14 day granulomas Centrifugation in 5—20 % sucrose in 0 01 M sodium acetate solution at 4 — *Upper left figure* 0 5 mg of HMW RNA, centrifuged for 9 h at 30 000 rpm — *Upper right figure* 0 4 mg of HMW RNA centrifuged for 20 h at 30 000 rpm The 28S component had already sedimented to the bottom of the tube — *Lower figures* 16S and 22S component were isolated from three runs and processed as described on page 33 and the isolated fractions were rerun separately at 30 000 rpm for 20 h

Table 5 Amino acid acceptor capacities of nucleic acid fractions extracted by the DOCA phenol method from 14 day granulomas

Enzyme preparation mg	Reaction mixture			Radioactivity (cpm) precipitated with TCA after incubation
	ATP μM	Buffer ml	Nucleic acid fraction μg	
1 0	10	0 4	LMW RNA 30	16 400
0 5	10	0 5	LMW RNA 30	11 800
1 0	10	0 4	LMW RNA 2	2 260
1 0	10	0 4	LMW RNA 1	580
1 0	10	0 4	HMW RNA 150	240
1 0	10	0 4	DNA 130	1 1[illegible]0
0	10	0 6	LMW RNA 28	190
1 0	10	0 5	—	340
1 0	—	0 5	LMW RNA 30	520

The experiment was performed as described on page 26 [illegible] μCi of ^{3}H lysine was added to each reaction mixture The results are means of three determinations

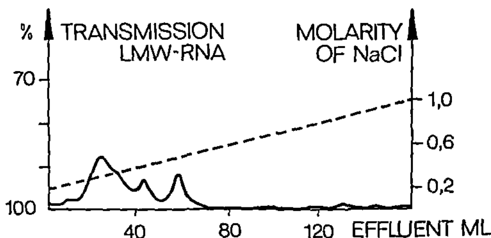


Fig 9 Chromatography of LMW RNA from 14 day granulomas on a methylated albumin kieselguhr column The sample (0 4 mg) was dissolved in 0 1 M NaCl 0 05 M phosphate buffer pH 6 7 Elution was performed with a linear concentration gradient of NaCl from 0 2 M to 1 0 M in the phosphate buffer The eluting concentrations of NaCl are first peak 0 34 M second peak 0 40 M, and third peak 0 48 M

22S fractions are seen in Fig 10 The 5S and 12S fractions gave more complex patterns in similar runs It was estimated that the fraction 28S represented 6[illegible] % of the total HMW RNA the fraction 16S about 30 %, the fraction 22S 3 % and the fractions 5S and 12S together about 3 %

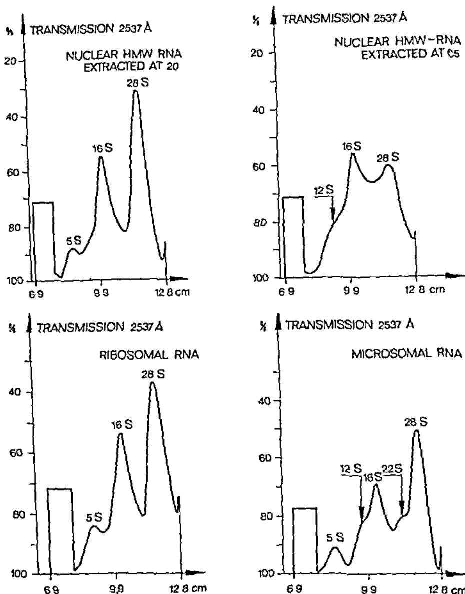


Fig 11 Sucrose density gradient analys s of PNA from subc llular fractions of 14 day granulomas Centrifuged in 5— 0 % sucro e gradient in 0 1 sodium acetate solution at 30000 rpm for 9 h at 4

The macromolecular characteristics of subcellular RNA were studied by sucrose density gradient ultracentrifugation The subcellular fractions were obtained from 45 granulomas 14 days old The crude 600 *g* sediment was used as a source of nuclear RNA The nuclear RNA were fractionated

into nuclear sap RNA by extraction at 20° (this fraction contained also the RNA of unbroken cells) and nucleolo chromosomal RNA by extraction at 65° Mitochondria were sedimented at 8000 g as described above, micro somes at 35 000 g for 30 minutes and ribosomes at 150 000 g for 1 hour The ribosomal RNA in this case represent only the RNA of free cyto plasmic ribosomes

The sucrose density gradient patterns are shown in Fig 11 The nuclear HMW RNA extracted at 20° and the RNA of free ribosomes resemble each other as both gave prominent peaks at 5S, 16S and 28S and little material between these peaks The 22S and 12S fractions of HMW RNA seem to be located in microsomes The nucleolo chromosomal HMW RNA gave a pattern with a prominent 16S peak and much material between 16S and 28S All these patterns were reproducible Difficulties were however, met in the analysis of mitochondrial RNA The 8000 g sediment contained two kinds of particles When the sediment was analysed in a sucrose density gradient (30 %—65 %) a light band was observed that contained much RNA and was devoid of cytochrome oxidase and a heavy band which had a low RNA content, but contained cytochrome oxidase The latter apparently contained also mitochondria The RNA extracted from the mitochondria gave variable polydisperse patterns The lighter material consisted of heavy microsomes since the RNA resembled those of microsomes sedimenting at 35 000 g

So called subribosomal particles, which are considered to be precursors of ribosomal subunits were isolated by zonal centrifugation (Fig 12)

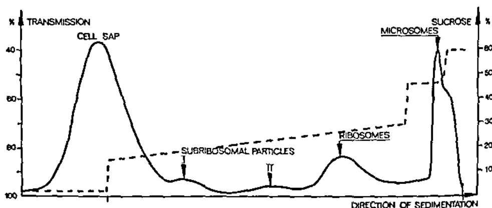


Fig 1° Fractionation of the post mitochondrial supernatant of 14 day granuloma homogenate in a onal rotor The supernatant SIII was obtained as described in Fig 3 Centrifugation in the zonal rotor took place at ?1000 rpm at about 4 for 9 h Continuous line transmission at 2537 A broken line sucrose concentration

RNA OF SUBRIBOSOMAL PARTICLES

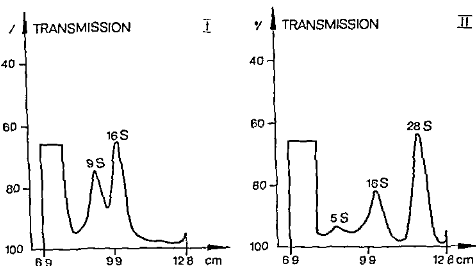


Fig 13 Sucrose density gradient analysis of RNA from subribosomal particles The particles I and II (see Fig 1) were precipitated with two volumes of ethanol at —5 overnight RNA was extracted by the DOCA phenol method (Fig 1) 5—20 % sucrose gradient in 0 1 M sodium acetate centrifugation at 30000 rpm at 4 for 8 h

Their RNA were analysed by sucrose density gradient ultracentrifugation (Fig 13) The heavier particles (II) resemble ribosomes in this respect while the lighter ones have a unique RNA pattern

Rapidly labelled RNA in 14 day granulomas

In order to study the labelling of RNA in 14 day granulomas ^{32}P phosphate was injected intraperitoneally into rats and the granulomas were removed after periods of ¼ 1 2 4½ and 12 hours and the nucleic acids were extracted at 20° by the DOCA phenol method There were four animals (16 grams of granuloma tissue) in each group

The distribution of radioactivity among nucleotides after various incorporation periods is seen in Table 5 Possible contamination of UMP by ^{32}P orthophosphate was checked by anion exchange and paper chromatography Non radioactive carrier was added to each radioactive UMP sample before the specific activity was determined The solution (pH 9) was then applied to a Dowex 1 column (1 × 2 cm) which was eluted successively with water (10 ml) 0 3 N formic acid (30 ml) which eluted two small UV absorbing fractions and 0 5 N ammonium formate which

eluted UMP, the specific activity of which was the same after as before chromatography Portions of UMP samples were also chromatographed together with UMP and radioactive phosphate on Whatman No 1 paper with 2 propanol hydrochloric acid The R_f values were 0 77 for UMP and 0 84 for ³²P phosphate All radioactivity from ³²P labelled UMP fractions was in the spot containing the carrier UMP When ³²P phosphate was mixed with the carrier UMP, only a trace of radioactivity was observed in the UMP spot

The distribution of radioactivity in the material resolved by a sucrose gradient after various incorporation times is seen in Fig 14 The label is first detected in fractions 5—12S and about 23S the latter becomes prominent after 2 hours incorporation and a new peak appears in the 16S region After 4½ hours the radioactivity curve begins to follow the UV transmission curve and after 12 hours the curves are identical A small peak in radioactivity is also observed in the fraction at about 35S after 1 2 and 4½ hours

The rapid changes in the distribution of radioactive matter among the nucleotides suggest that ribonucleic acids with different nucleotide compositions are synthesized Synthesis of ribosomal RNA starts to dominate the pattern after 4½ hours as revealed both by the distribution of radioactivity among the nucleotides and by the sucrose density gradient patterns

In order to study further the rapidly labelled RNA 10 mCi of radio active phosphate was injected intraperitoneally into two rats bearing five granulomas each Two hours later the granulomas (10 g of tissue) were

Table 5 Incorporation of ³²P phosphate in vivo into HMW RNA extracted from 14 day granulomas at °0

…rpor…on …ne	Specific activity of RNA (cpm/mg)	Specific activities of nucleotides (cpm/µM)				Distribution of ³²P phosphate among nucleotides (%)				
		CMP	AMP	UMP	GMP	CMP	AMP	UMP	GMP	(CMP+GMP)/(AMP+UMP)
[illegible]	8100	930	1_60	40_0	1_0	21 9	14 6	39 3	_5	0 9
2	11160	16_0	1650	40_0	16_0	23 _	14 7	3_ 6	28 0	1 0
½	18__0	[illegible]0	8060	10__0	64_0	30 1	1 6	24 0	27 8	1 4
2	31100	10040	11500	10400	9 _0	_7 _	21 6	19 4	31 3	1 4

HMW RNA were hydrolysed in 0.3 N KOH at 37° for _0 h, nucleotides were separated by combined cation an anion exchange chromatography The distribution of radio activity among nucleotides is expressed by the total ³²P counts for a given nucleotide as a percentage of the sum of activities of all nucleotides in the RNA preparation

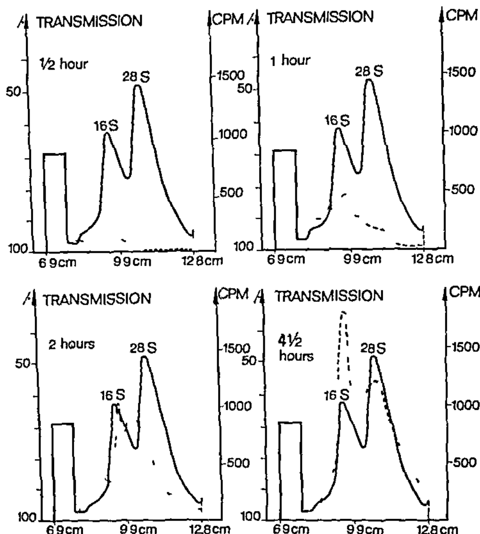


Fig 14 Incorporation of ^{32}P phosphate into HMW RNA from 14 day granulomas Continuous line = transmission at [illegible] Å Broken line = ^{32}P activity The original radioactivity patterns are superimposed on the optical patterns (1 1 mg of HMW RNA centrifuged in 5—20 % sucrose gradient in 0 01 M sodium acetate solution at 21 000 rpm at 4° for 17 h)

removed and the nucleic acids were extracted at 20° and 65° as described in the methods section

The specific activities of the fractions were measured before and after filtration through Sephadex G 25 The specific activities of the HMW RNA fractions were the same before and after the gel filtration The DNA—LMW RNA fractions contained radioactive contaminants of low

molecular weight The specific activities after gel filtration are presented in Table 6

The HMW RNA extracted at 65° are most rapidly labelled They include according to Georgiev *et al* (1963) RNA of nucleoli, which are the sites of rRNA synthesis and chromosomal RNA, which are mRNA The distribution of ^{32}P phosphate among nucleotides (Table 6) suggests that the synthesis of rRNA predominates The ratio $\frac{CMP + GMP}{AMP + UMP}$ is 1 4, the corresponding ratio is 1 8 for rRNA while for DNA, $\left(\frac{C + G}{A + T}\right)$ is 0 8 (Table 3) HMW RNA extracted at 20° is more 'DNA like whereas LMW RNA have a high degree of labelling in CMP This

Tables 6—7 Rapidly labelled RNA fractions from 14 day granulomas Labelling 10 mCi of ^{32}P phosphate per rat for 2 h

Table 6 Specific activities and distribution of ^{32}P phosphate among nucleotides

RNA fraction	Specific activity cpm/mg	Distribution of ^{32}P phosphate (%)				
		CMP	AMP	UMP	GMP	$\frac{CMP+GMP}{AMP+UMP}$
HMW RNA extracted at 20	155 800	24 7	14 0	36 3	25 7	1 0
LMW RNA	363 600	59 2	12 7	12 7	15 4	[illegible]1
HMW RNA extracted at 65	1 401 200	29 3	21 1	20 5	29 1	1 4

Distribution of ^{32}P phosphate among nucleotides was determined by combined cation and anion exchange chromatography Values are means of two determinations

Table 7 Formation of DNA RNA hybrids

Sample	Total cpm in RNA	cpm in DNA RNA hybrids			
		Experiment 1		Experiment 2	
		cpm	% of total	cpm	% of total
HMW RNA extracted at 20	8 320	4?3	5 7	392	4 7
HMW RNA extracted at 65	1 018	184	18 1	194	19 0
tRNA	464	48	10 3	49	10 6

HMW RNA were obtained by precipitation with 3 M sodium acetate tRNA by MAK chromatography (see Fig 9) DNA were extracted from 14 day granulomas by the DOCA phenol method

apparently is due to the rapid turnover rate of the pCpA terminus of tRNA (Hiatt 1962)

The formation of hybrids by RNA and DNA has been used to determine the amount of mRNA in rapidly labelled RNA (Spiegelman and Hayashi 1963 Armstrong and Boezi 1965) The method is based on the assumption that mRNA forms a double stranded RNAase resistant structure with DNA that has a similar nucleotide sequence when they are heated and annealed together

As already mentioned the experiment was performed as described by Armstrong and Boezi (1965) Preliminary experiments indicated that 0 1 % of the radioactive RNA was absorbed on a Millipore filter when the hybridization procedure was performed without heat denatured DNA present The results of the experiments, which are presented in Table 7 indicate that under comparable conditions the HMW RNA extracted at 65° are hybridized most Since the tRNA fraction also formed hybrids it apparently contained besides transfer RNA also mRNA

The sucrose density gradient pattern of the HMW RNA extracted at 65° is seen in Fig 15 The radioactivity follows the optical pattern suggesting uniform labelling of the RNA

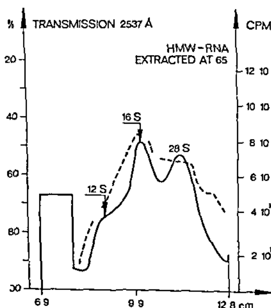


Fig 15 Sucrose density gradient analysis of HMW RNA extracted at 65 from 14 day granulomas after labelling with P phosphate for • h in vivo Continuous line transmission Broken line radioactivity 5— 0 % sucrose in 0 01 M sodium acetate solution centrifugation at 30 000 rpm for 11 h at 4

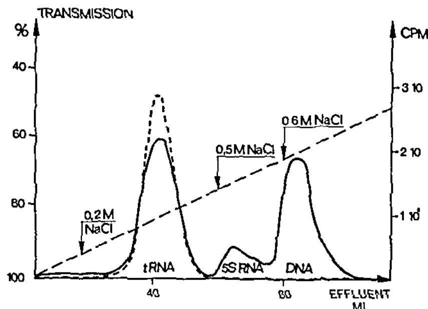


Fig 16 MAK chromatography of DNA—LMW RNA fraction of 14 day granulomas after incorporation of ³²P phosphate in vivo for 2h The nucleic acids were passed through Sephadex G 25 in 0 1 M NaCl—0 02 M phosphate pH 6 7 before chromatography 2 mg of nucleic acids was applied and eluted as indicated in the figure Continuous line per cent transmission at 253 m Broken line radioactivity

The DNA—LMW RNA fraction was chromatographed on a MAK column (Fig 16) All the radioactive matter was in the first fraction The 5S ribosomal RNA and DNA were not labelled in two hours

No prominent radioactive RNA fraction sedimenting more rapidly than 28S was observed in density gradients when sedimentation was carried out in 0 01 M sodium acetate solution (Fig 17) Other investigators (Hiatt 1962 Scherrer and Darnell 1962 Wilkinson and Kirby 1966 Parish and Kirby 1967) have reported rapidly labelled RNA that sediment at 35—45S They all used higher salt concentrations (0 05 M or higher) in their gradients The effect of salt concentration on the sedimentation pattern was studied by running HMW RNA extracted from 14 day granulomas at +20° in 0 01 M and 0 1 M sodium acetate solutions (Fig 17) It can be seen that a considerable portion of the radioactive matter sediments faster than the 28S RNA in the higher salt concentration The effect of salt concentration was also seen in the distribution of ³²P phosphate among nucleotides (Table 8) It seems that the fraction sedimenting at 20—24S is not affected but the fraction sedimenting faster than 28S in 0 1 M sodium acetate solution is probably derived from the fraction sedimenting at 1—12S in 0 01 M sodium acetate solution

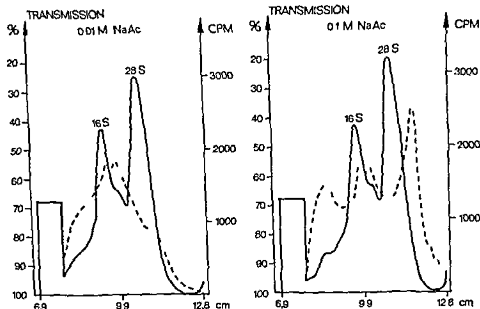


Fig 17 Effect of sodium acetate concentration on the sedimentation of rapidly labelled P N 1 from 14 day granulomas in sucrose density gradients The runs were performed simultaneously (5—20 % sucrose 21 000 rpm for 17 h) Continuous line % transmission at 2537 Å Broken line radioactivity Radioactive HMW RNA (30 000 cpm) were applied onto the gradient made in 0 01 M sodium acetate solutions 41 % of the radioactivity was in the region 1—15S *after centrifugation 30 % between 16S and 28S* while 29 % sedimented at 28S or faster Radioactive HMW RNA (40 000 cpm) were applied onto the gradient in 0 1 M sodium acetate solution The corresponding distribution was 31 % 24 % and 44 %

Table 8 The effect of salt concentration on sedimentation fractions of rapidly labelled HMW RNA extracted from 14 day granulomas at 40 as revealed by distribution of P phosphate among nucleotides

Nucleotide	Sedimentation in 0 01 M sodium acetate			Sedimentation in 0 1 M sodium acetate		
	1—15S	16—28S	over 28S	1—15S	16—28S	over 28S
CMP	23 1	3 [illegible]	—	4[illegible] 2	[illegible]7	29 1
AMP	14 3	5 8	—	13 2	9 3	20 4
UMP	30 6	45 7	—	28 6	4[illegible] 3	2[illegible] 4
GMP	31 9	44 9	—	15 9	42 [illegible]	23 1

Values are percentages of the sum of the activities in the nucleotides RNA were labelled for [illegible] h in vivo RNA fractions were obtained from similar density gradient runs as in Fig 17 by precipitation together with carrier RNA with [illegible] % TCA The distribution of radioactivity was determined after alkaline hydrolysis and separation of the nucleotides by [illegible] chromatography on cation and anion exchange columns

Discussion of the extraction and fractionation of nucleic acids

The problem with the extraction of nucleic acids is to free them from associated proteins and lipoprotein membranes and simultaneously to protect them from nucleases. The introduction of phenol (Kirby 1956) first made it possible to extract high molecular weight ribonucleic acids from mammalian tissues. Phenol denatures proteins and inactivates nucleases rapidly although some of the latter apparently survive the extraction and become active after the removal of the phenol (Huppert and Pelmont 1963). The introduction of lipophilic salts and chelating agents led to an increased yield of nucleic acids (Kirby 1962), especially of DNA, probably as they disrupt the cation linkages in lipoprotein membranes (Kirby 1964). The combination of sodium deoxycholate with sodium naphthalenedisulphonate and phenol was better for the extraction of nucleic acids from granulomas than the use of sodium dodecyl sulphate instead of sodium deoxycholate with sodium naphthalenedisulphonate. Apparently SDS releases some ribonucleases from granulomas that cannot be removed completely by treatment with sodium chloride and phenol. Smirnov *et al.* (1964) used SDS with phenol for the extraction of RNA from Ivalon sponge granulomas but they do not describe their RNA preparation.

The fractionation of the nucleic acid preparations proved difficult in the present study. One possibility was preliminary subcellular fractionation, which however proved impractical for routine use because of the low yield of RNA. Granulation tissue in implants contains besides cells abundant amounts of extracellular collagen fibres and sponge network. To break these structures the homogenization must be forceful, which apparently disrupts subcellular structures.

The salting out procedure combined with Sephadex G 200 fractionation produced three nucleic acid fractions. Some workers (Alexander *et al.* 1967, Williamson and Kirby 1966) have observed contamination of the HMW RNA fraction by DNA when the nucleic acid preparation was extracted with 3 M salt solution. It also has been demonstrated in Kirby's laboratory that the salting out procedure is an efficient method for removing DNA. When rat hepatomas were labelled with ^{14}C thymidine the HMW RNA isolated from the tumours were not radioactive (Ahonen and Kirby, unpublished).

The nucleic acid fractions. The base composition of the DNA (Table 2) follows quite closely the so called Chargaff rules (Chargaff 1955). The values in this study should be compared with those of Wyatt (1950), who used methods similar to those used in this work for the hydrolysis and

chromatographic separation of the bases He obtained the values C 20 5 % A 28 7 % T 28 5 % and G 21 5 % for rat bone marrow DNA The granuloma DNA had slightly higher C and C contents, this might have been due to incomplete extraction of DNA by the DOCA phenol method

In this work the term LMW RNA has been used for the RNA fraction that is soluble in 3 M sodium acetate and diffuses into the inner volume of Sephadex G 200 gel particles The nucleotide composition of this fraction (Table 2) differs from the nucleotide compositions of total HMW RNA and rRNA The typical features are the presence of 5 ribosyluracil 2′(3) monophosphate and higher contents of GMP and CMP The LMW RNA fraction contains other RNA than transfer RNA as shown by the nucleotide composition (low 5 ribosyluracil 2 (3′) monophosphate content) the presence of guanosine (in addition to adenosine) as terminal nucleoside and the incomplete reversibility of hyperchromicity (Brown 1963) Apparently ribosomal 5S RNA are present (Rosset *et al* 1964) The 5S RNA are eluted from a MAK column by 0 50 M sodium chloride solution (Galibert *et al* 1965) and were present in the third fraction in Fig 9

The labelling of the LMW RNA fraction after 2 hour incorporation of ^{32}P phosphate is partly due to the turnover of the pCpCpA terminus of tRNA (Hiatt 1962) Since considerable activity was present also in GMP and UMP, it is likely that some newly formed RNA were present Possibly they were incomplete or broken mRNA that were either being synthesized at the time of removal of tissue or broken (on homogenization) mRNA strands The presence of mRNA in the LMW RNA fraction was also suggested by the hybridization experiments (Table 7)

The HMW RNA fraction in rat liver consists of ribosomal RNA and so called rapidly labelled RNA (Kirby 1965 Hastings and Kirby 1966) The nucleotide composition of HMW RNA from granulomas resembles the nucleotide compositions of rRNA and total RNA (Table 2) and that of HMW RNA from rat liver (CMP 31 4 AMP 18 5 UMP 17 3 and GMP 32 8 mole % Kirby 1965)

Ribosomal RNA in multicellular organisms consist of two main types of molecules a so called 18S component (mol wt around 5 10^5) and a so called 28S component (mol wt of the order of 1 5 10^6, Spirin 1964) The sedimentation values in the literature range from 16 to 18S and from 24 to 30S according to the conditions Divalent cations in particular affect the $s^0_{20,w}$ values (Spirin 1964) The results obtained in this investigation closely resemble those of Petermann and Pavlovec (1963) who obtained for rat liver rRNA the values 16 0S and 27 9S in 0 1 M sodium chloride—0 01 M sodium acetate of pH 4 8 in the absence of divalent cations The minor components observed in this study (5 12 and 22S)

apparently were not due to the degradation of 16S and 28S RNA by RNAse because they were constantly present in similar ratios. The 22S component has been found by Petermann and Pavlovec (1963) in rat liver rRNA and by Hastings *et al.* (1965) in HMW RNA from rat liver prepared by the method II of Kirby (1965). In granulomas this fraction is located in the microsomes.

The yield of RNA extracted at 20° was around 90 % of the total RNA content and could be slightly increased by extraction at 65°; the latter RNA were more rapidly labelled by ^{32}P than the former. This is in accord with the result of Georgiev *et al.* (1963), but contrary to the observation of Hiatt (1962) who found that the specific activity of RNA isolated from regenerating liver by SDS phenol at 20° was the same as that of the RNA extracted by the Schmidt-Thannhauser method.

Rapidly labelled RNA. In 1957 Volkin and Astrachan observed that following phage infection of a bacterium a RNA fraction appeared that had a very rapid turnover. Later Jacob and Monod (1961) presented their hypothesis that a special RNA called messenger RNA mediates the genetic information for protein synthesis to the ribosomal template. Little success has been achieved to date in the characterization of natural mRNA.

It has been established that not all RNA that become labelled before the bulk ribosomal RNA are mRNA. For instance, Armstrong and Boezi (1964) found that only 25 % of the rapidly labelled RNA in *E. coli* cells forms hybrids with homologous DNA. On the other hand, at least some mRNA may have a lower turnover (Singer and Leder 1966). It has been suggested that in rapidly dividing cells such as HeLa cells (Scherrer and Darnell 1962) and regenerating liver cells (Steele, Okamura and Busch 1966), a considerable proportion of the rapidly labelled RNA consists of precursors of rRNA.

In this study conventional methods were used in the analysis of rapidly labelled RNA. The incorporation of ^{32}P phosphate *in vivo* is analogous with the pulse labelling experiments used in bacterial systems (Hiatt 1962). The sequence of the labelling of the various RNA fractions resolved by sucrose density gradients indicated that the label was first present in RNA different from ribosomal 16S and 28S RNA. Of interest is the finding that no prominent component sedimenting faster than 28S RNA was observed when the salt concentration in the sucrose density gradients was low. It is possible that the 35—55S RNA (Scherrer and Darnell 1962, Steele *et al.* 1966) may have been artefacts produced by the aggregation of rapidly labelled RNA with rRNA (Parish and Kirby 1966, Fig. 17).

Nucleic acids during different phases of development of granulomas

Incorporation of ^{14}C glycine in vitro

The experiments with ^{14}C glycine were planned to obtain information on the biosynthetic capacities of granulation tissues of different ages. The specific and total activities of DNA, RNA, collagen and other proteins were determined after incubating granulation tissue slices in the presence of ^{14}C glycine.

Preliminary tests showed that granuloma tissue slices respired in the incubation medium for at least 6 hours. No differences in the specific activities of RNA nucleotides were observed when the gas phase was air or 90 % O_2—5 % CO_2. About 2 % of the added ^{14}C glycine was incorporated into nucleic acids and proteins in 4 hours.

Three series of experiments were performed. In two series only the

Table 9. Incorporation of ^{14}C glycine into nucleic acids of granulomas of different ages in vitro

Nucleic acid	Age of granulomas (days)					
	5	7	14	21	28	60
DNA						
Specific activity (dpm/mg)	6[illegible]0	2160	530	160	160	110
Total activity (dpm/g of tissue wet weight)	420	1530	640	190	190	60
RNA						
Specific activity of AMP (dpm/μM)	4410	4890	4830	28[illegible]0	1980	380
Specific activity of GMP (dpm/μM)	2[illegible]90	2160	2530	1[illegible]30	1900	370
Total activity of RNA (dpm/g of tissue wet weight)	5400	7100	13900	9600	2[illegible]00	2200
Ratio of total activities						
DNA/RNA	0 08	0 216	0 046	0 0[illegible]0	0 086	0 027

Granuloma slices were incubated with ^{14}C glycine for 4 h. Nucleic acids were extracted by the DOCA phenol method and RNA and DNA were separated by alkaline hydrolysis. Specific activities of AMP and GMP were determined after chromatography on anion exchange resin. The specific activity of DNA was determined after acid hydrolysis.

specific activity of RNA was measured. In the third series also the specific activities of DNA, collagen and other proteins were determined.

The specific and total activities of the nucleic acids are presented in Table 9. The total activity was obtained by multiplying the specific activity by the amount of the nucleic acid.

The results show that DNA is for the most part synthesized during the first two weeks of granuloma development. It is rather surprising, however, that radioactivity was present in DNA even in the granulomas removed on the 60th day. Contamination of the sample by protein is unlikely since the UV absorbance ratios of the DNA hydrolysates agreed well with the base composition (Fredericq, Oth and Fontaine 1961). The results show that most of the cells in a granuloma are formed during the first two weeks and that proliferation then continues at a much reduced rate.

Table 10. Incorporation of C glycine into proteins of granulomas of different ages in vitro

Protein	Age of granulomas (days)					
	5	7	14	21	28	60
Collagen						
Specific activity (dpm/mg N)	—	7790	7420	4440	5240	1300
Total activity (dpm/g of wet tissue weight)	—	3500	12600	11600	5200	4300
Non collagenous proteins						
Specific activity (dpm/mg N)	530	21960	29980	23490	19[illegible]60	6530
Total activity (dpm/g of wet tissue weight)	—	107900	119500	1[illegible]0100	47700	24700
Ratios of total activities						
Collagen/non collagenous protein		0.03	0.10	0.09	0.10	0.17
RNA/collagen		2.0[illegible]	1.10	0.83	0.43	0.51
DNA/non collagenous protein		0.06	0.1[illegible]	0.03	0.05	0.09

Granulomas [illegible] were incubated with C glycine for 4 h. Collagen was recovered from the nucleic [illegible] extraction residue with 10% TCA at 90°. Non collagenous proteins were solubilized from the residue with alkali. The specific activities were determined after hydrolysis.

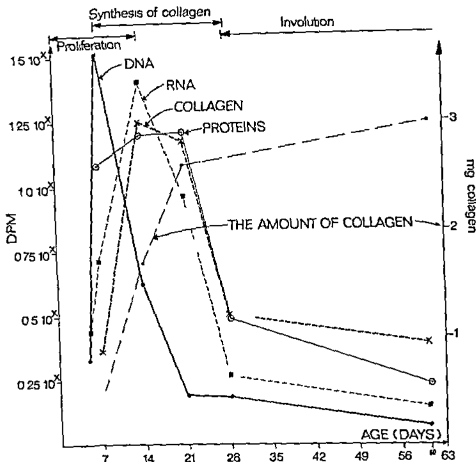


Fig 18 Development of experimental granuloma as indicated by the incorporation of C glycine into nucleic acids and proteins The curves plot the total dpm per g of tissue (wet weight) and the amount of collagen in mg of N per g of tissue (wet weight) Developmental phases are indicated in the upper part of the figure The value of x is 3 for DNA 4 for RNA 5 for proteins 4 for collagen

The results on the incorporation of [14]C glycine into proteins are presented in Table 10 The purity of the collagen fraction was checked by analysis of hydroxyproline and total nitrogen The ratio of hydroxyproline nitrogen to total nitrogen varied between 0 10 and 0 08

The reduction of the specific activity of collagen with increasing granuloma age is due to the dilution of newly formed by pre existing collagen The maximal incorporation of [14]C glycine in collagen occurs during the third week long after the incorporation in DNA

specific activity of RNA was measured In the third series also the specific activities of DNA collagen and other proteins were determined

The specific and total activities of the nucleic acids are presented in Table 9 The total activity was obtained by multiplying the specific activity by the amount of the nucleic acid

The results show that DNA is for the most part synthesized during the first two weeks of granuloma development It is rather surprising however, that radioactivity was present in DNA even in the granulomas removed on the 60th day Contamination of the sample by protein is unlikely since the UV absorbance ratios of the DNA hydrolysates agreed well with the base composition (Fredericq Oth and Fontaine 1961) The results show that most of the cells in a granuloma are formed during the first two weeks and that proliferation then continues at a much reduced rate

Table 10 Incorporation of C glycine into proteins of granulomas of different ages in vitro

Protein	Age of granulomas (days)					
	5	7	14	[illegible]1	28	60
Collagen						
Specific activity (dpm/mg N)	—	7[illegible]90	7420	4440	[illegible]40	1300
Total activity (dpm/g of wet tissue weight)	—	3500	12600	11600	[illegible]900	4300
Non collagenous proteins						
Specific activity (dpm/mg N)	530	21960	29980	23420	19260	6[illegible]30
Total activity (dpm/g of wet tissue weight)	—	107900	119[illegible]00	120100	4[illegible]00	[illegible]4700
Ratios of total activities						
Collagen/non collagenous protein		0.03	0.10	0.09	0.10	0.1
DNA/collagen		2.02	1.10	0.83	0.43	0.[illegible]1
DNA/non collagenous protein		0.06	0.12	0.03	0.0[illegible]	0.09

Granulomas [illegible] were incubated with C glycine for 4 h Collagen was recovered from the nucl[illegible] [illegible] extraction residue with 10 % TCA at 90 Non collagenous protein were solu[illegible] [illegible] from the residue with alkali The specific activities were determined after hydr[illegible]

proteins are not equal, the ratio of the amount of collagen synthesized to the amount of other proteins synthesized cannot be determined

The conclusion that can be drawn from these experiments (Fig. 18) is that most of the DNA are synthesized during the first 14 days. Cell proliferation is accompanied and followed by high rates of synthesis of RNA and non collagenous proteins. The synthesis of collagen starts after the first week, is maximal during the third week, and then decreases rapidly.

In order to get information about the incorporation of ^{14}C glycine into ribosomes and polysomes the following experiment was performed. Slices of 21 day granulomas were incubated with ^{14}C glycine for 2 hours as described earlier. The slices were frozen in dry ice acetone after removing the incubation medium and pressed through an aperture 0.6 mm in diameter. Two millilitres of 0.01 M Tris buffer of pH 7.4 that was 0.0015 M in $MgCl_2$ and 0.01 M in KCl was added to the homogenate and the mixture was centrifuged at 8000 g and 0° for 15 minutes. A portion of the supernatant was layered on a 10—30 % sucrose gradient in the above mentioned buffer. After centrifugation at 21000 rpm and 0° for 3 h in a swing out rotor, the polythene tube was pierced with a hypodermic needle and 20 fractions were collected. The fractions were analysed for radioactivity, absorbance at 2600 Å and protein.

The results are presented in Fig. 19. Similar patterns were obtained whether bentonite and polyvinyl sulphate were present or not. An interesting feature is the relatively low yield of polysomes as compared with other tissues (*e.g.* Munro *et al.* 1964). No hydroxyproline was detected in the fractions that sedimented more rapidly than the monomeric (80S) ribosomes; this is in accord with the results of Kumento *et al.* (1966).

Nucleotide composition of total RNA

The nucleotide compositions and the amounts of total RNA are presented in Table 11. 5-Ribosyluracil 2′(3′) monophosphate combined with uridylic acid was present even in the oldest granulomas. Cytidine, adenosine, uridine and guanosine were detected in the chromatograms but their amounts were not determined.

No changes were noted in the nucleotide compositions. Also the ratio $\frac{GMP + CMP}{AMP + UMP}$ remained constant at a high value as in the case of HMW RNA and rRNA (Tables 2 and 4).

Table 11 *Nucleotide compositions of total PNA*

Nucleotide	Age of granulomas in days				
	7 (4)	14 (5)	21 (5)	35 (5)	90 (4)
CMI	31 4±0 4	31 2±0 9	31 4±0 4	31 0±0 8	31 9±0 7
AMP	18 6±0 9	18 5±0 7	18 0±0 8	19 1±0 1	18 1±0 9
UMP	17 7±0 8	17 6±0 7	17 3±0 5	17 2±0 2	17 6±0 8
CMP	32 3±0 9	32 6±0 7	33 3±0 6	3 0±0 7	3_ 4±0 7
CMP+GMP / AMP+UMP	1 8	1 8	1 8	1 7	1 8
The amount of RNA (mg/100 mg of viscose cellulose)	2 0±0 3	4 2±0 2	4 6±0 1	4 6±0 4	3 2±0 4

The values are molc percentages PNA was extra ted by the Schmidt Thannhauser procedure and the nucleotide compositions determined by chromatography on anion exchange resin The amount of PNA was calculated from the amounts of individual nucleotides The values are means ± S D The number of experiments is indicated in parentheses

Nucleic acid fractions

Extractability of nucleic acids Nucleic acids were extracted from pooled granulomas by the DOCA phenol method (Fig 1) The amounts of residual nucleic acids were determined by the Schmidt Thannhauser method (page 17) The yield of RNA extracted at 20 ° varied from 68 to 92 % but the proportion of extractable DNA varied as follows

7 day granulomas	80 % of the total DNA
14 day granulomas	54 % of the total DNA
21 day granulomas	58 % of the total DNA
35 day granulomas	40 % of the total DNA

The base composition of the extracted DNA was constant $\left(\frac{G+C}{A+T} = 0\ 8\right.$ $\left.\frac{C+T}{A+G} = 1\ 0\right)$

Nucleotide composition of LMW RNA LMW RNA which, as stated before include tRNA 5S rRNA and rapidly labelled hybridizable RNA were isolated as described in Fig 1 from pooled granulomas (16 to 40 in each age group) and four rat livers The nucleotide composition was

determined by chromatography on anion exchange resin after hydrolysis in all alkali. The results are presented in Table 12.

Clear differences can be observed between the granulomas 14, 21 and 37 days old that produced collagen at a high rate and the 7 day and 60 day granulomas. The LMW RNA from rat liver resemble those from the 7 day granulomas. The characteristic feature of LMW RNA during rapid collagen synthesis is the high content of CMP and GMP. If it is assumed that LMW RNA include mRNA, the results suggest that mRNA for collagen synthesis would be in the LMW RNA of collagen synthesizing granulomas. Collagen contains considerable amounts of proline and glycine, the suggested codes for which are CCX and GGX. Corresponding anticodons in tRNA would be GGX and CCX. Thus an increase in the amounts of proline and glycine specific tRNA should also raise the ratio $\frac{CMP + GMP}{AMP + UMP}$

Table 12. Nucleotide compositions of LMW RNA

Nucleotide	Age of granuloma (days)					Rat liver LMW RNA
	7	14	21	37	60	
CMP	29.6	31.7	34.1	34.1	29.9	29.0
AMP	17.1	14.7	14.0	15.4	20.6	18.3
UMP	16.8	16.7	14.0	15.4	20.6	16.5
GMP	33.7	35.0	35.4	32.7	27.7	33.2
φ UMP	2.4	1.8	2.5	2.2	1.0	3.0
$\frac{CMP+GMP}{AMP+UMP+\varphi\ UMP}$	1.7	2.0	2.3	2.0	1.4	1.6

The values are mole precentages. RNA were extracted at 20° by the DOCA phenol method and LMW RNA separated from DNA by gel filtration in Sephadex G-200 column. Nucleotide compositions were determined by chromatography on anion exchange resin. The values are means of two determinations on the same samples.

HMW RNA during the phases of proliferation and collagen synthesis. HMW RNA extracted at 20° include besides rRNA rapidly labelled hybridizable RNA which are presumably mostly cytoplasmic mRNA. The HMW RNA extracted at 65° include nucleolar RNA which are thought to include the precursors of rRNA and chromosomal RNA which apparently are mostly nuclear mRNA. In 6 day granulomas the synthesis of DNA is rapid, whereas in 18 day granulomas it has slowed down (Fig. 18). Consequently we would expect changes in the relative proportions of nucleolar and chromosomal RNA, especially as the protein synthesis is still rapid in 18-day granulomas (Fig. 18).

Table 13 *Nucleotide compositions of HMW RNA from 6 and 18 day granulomas*

Preparation	Nucleotide				CMP+GMP / AMP+UMP
	CMP	AMP	UMP	GMP	
HMW RNA (20)					
6 day granulomas	32 0	18 0	17 5	32 5	1 8
18 day granulomas	31 9	18 2	17 4	32 5	1 8
HMW RNA (65)					
6 day granulomas	30 0	19 7	20 1	30 2	1 5
18 day granulomas	30 7	18 0	17 5	33 8	1 8

The values are mole percentages and means of two determinations on two RNA preparation Cation exchange chromatography

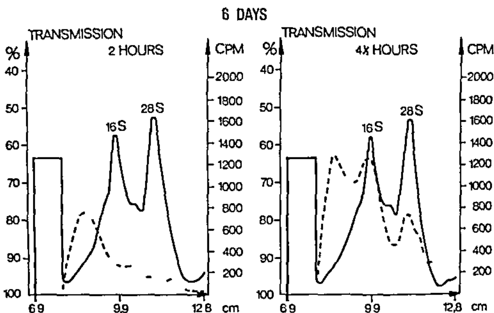


Fig 20 Incorporation of ³²P phosphate into HMW RNA extracted at +20 from 6 day granulomas Incorporation times are indicated in the figure 5—20 % sucrose in 0 01 M sodium acetate solution centrifugation at 30 000 rpm for 9 h at 4 Continuous line per cent transmission at 2537 Å Broken line radioactivity

The sucrose density gradient patterns of HMW RNA extracted at 20° from 6 day and 18 day granulomas are similar The relative proportions of the components (5S 12S, 16S 22S and 28S Fig 10) were the same in both as in 14 day granulomas (page 34) The nucleotide compositions did not differ either (Table 13)

Distinct differences were observed both in the sucrose density gradient patterns (Fig 22) and in the nucleotide compositions of the HMW RNA

fractions extracted at 65° (Table 13) The ratio $\frac{CMP + GMP}{AMP + UMP}$ is 1 8 for rRNA and the ratio $\frac{C + G}{A + T}$ is 0 8 for DNA Thus the change indicates that the proportion of chromosomal or DNA like RNA is higher in 6 day than in 18 day granulomas

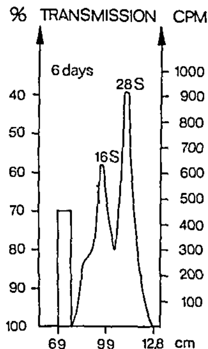
Rapidly labelled RNA in 6 day and 18 day granulomas were also studied First ^{32}P phosphate was injected intraperitoneally into two rats (1 mCi/rat) each bearing 6 day granulomas After incorporation for 2 and 4½ hours the granulomas were removed and the HMW RNA extracted at 20° The sucrose density gradient patterns are shown in Fig 20 Radio activity starts to follow the optical pattern after 4½ hours of incorporation and the distribution of ^{32}P phosphate among nucleotides (CMP 33 4 AMP 18 2 GMP 31 2) also suggests that mainly rRNA are synthesized In contrast radioactivity is detected only in the 5—14S region after two hours of labelling This experiment shows that 2 hour labelling *in vivo* is suitable for the study of rapidly labelled RNA also in 6 day granulomas since the synthesis of rRNA will not obscure the synthesis of mRNA

The patterns on sucrose density gradients (Fig 21) show that most of the rapidly labelled RNA in 6 day granulomas extracted at 20° sediment slower than 16S RNA whereas the incorporation of ^{32}P phosphate in 18 day granulomas mainly occurs into RNA sedimenting at 16—24S In collagen synthesizing cells one would expect to find mRNA of molecular weight 10^6 (corresponding to the α chain mol wt 90000) the S value for a poly nucleotide of this size should be about 22 (Spirin 1964) The distribution of ^{32}P phosphate among nucleotides (Table 14) does not indicate any differences in the rapidly labelled RNA extracted at 20°

Table 14 Comparison of HMW RNA from 6 day and 18 day granulomas Distribution of ^{32}P phosphate among nucleotides after labelling for 2 h in vivo

Preparation	Nucleotide				$\frac{CMP+GMP}{AMP+UMP}$
	CMP	AMP	UMP	GMP	
HMW RNA (20°)					
6 day granulomas	[illegible]	18 1	31 9	27 8	1 0
18 day granulomas	[illegible]	[illegible]	30 8	27 8	1 1
HMW RNA (65°)					
6 day granulomas	31 1	21 2	19 9	28 8	1 5
18 day granulomas	24 3	[illegible]	[illegible]	28 8	1 1

Labelling for 2 h *in vivo* The values are means of two determinations on two RNA preparations Successive chromatography on cation and anion exchange resin



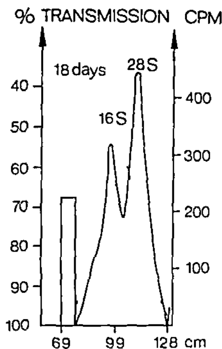
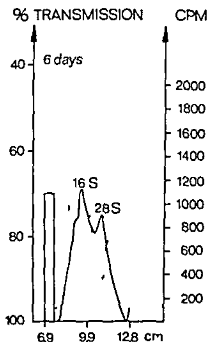
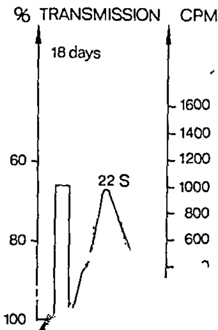


Fig 21





Fig

Figs 21— Comparison of the sedimentation 18 day granulomas lab lled with P phosphate in ucrose gra li nt in 0 01 M so lium a etate sol Continuous line t r cent tran mi ion at

Distinct differences were observed when rapidly labelled HMW RNA extracted at 65° were analysed (Fig 22 Table 14) The distribution of ^{32}P phosphate among nucleotides indicates that the proportion of DNA like rapidly labelled RNA is smaller in 6 day granulomas than in 18 day granulomas and the proportion of ribosomal precursors dominate in the former This is in contrast to the nucleotide compositions The nucleotide compositions however refer to the total nucleolo chromosomal RNA whereas the distribution of ^{32}P phosphate is a property of the rapidly labelled RNA that have a fast turnover (mRNA) or are transferred rapidly to the cytoplasm to form rRNA (ribosomal precursors) This result is in agreement with the data on ^{14}C glycine incorporation which indicate that the synthesis of rRNA slows down after 14 days of development On the basis of this interpretation the absence of the radioactive fraction sedimenting faster than 28S RNA (Fig 22) from 18 day granulomas means that the ribosomal precursors are of high molecular weight and/or have a different secondary structure than mature rRNA molecules A similar conclusion has been made in studies of other tissues too (Perry 1967)

Incorporation of labelled nucleosides into HMW RNA extracted at 20° HMW RNA rapidly labelled *in vivo* that were extracted at 20° suggested that a radioactive fraction equal in size to the mRNA for collagen synthesis was present in collagen synthesizing granulomas (Fig 21)

This observation was checked by labelling with another precursor of RNA *In vivo* labelling with ^{3}H uracil or ^{3}H cytosine was not successful since the specific activities were too low for measurement When tissue slices from 6 day and 18 day granulomas were incubated in the presence of ^{3}H uridine and ^{3}H cytidine it was observed that uridine is less rapidly incorporated than cytidine After 30 minute labelling with ^{3}H uridine *in vitro* the specific activity of HMW RNA extracted at 20° was 3210 cpm/mg on the 6th day and 1130 cpm/mg on the 18th day The corresponding values when ^{3}H cytidine was used were 30120 cpm/mg and 16810 cpm/mg Sucrose density gradient patterns of the ^{3}H cytidine labelled HMW RNA extracted at 20° are shown in Fig 23 A prominent radioactive fraction sedimenting at 22S is present in both 6 day and 18 day granulomas A similar result was obtained when the sedimentation analysis was performed in sucrose gradients in 0 01 M sodium acetate The same was also observed when ^{3}H uridine was the precursor The only difference in the sucrose density gradient patterns is that the RNA from 6 day granulomas included more radioactive RNA sedimenting faster than 28S than the RNA from 18 day granulomas

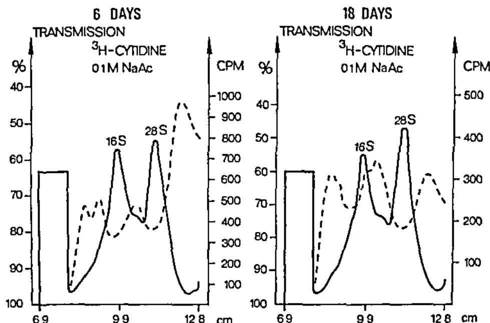


Fig 23 Comparison of the sedimentation patterns of HMW RNA extracted at 20 from 6 day and 18 day granulomas labelled with ^{3}H cytidine in vitro for 20 min 5—20 % sucrose in 0.1 M sodium acetate centrifugation at 30 000 rpm for 9 h at 4 Continuous line per cent transmission at 253 Å Broken line radioactivity

After granuloma slices were incubated in the presence of labelled nucleosides for one hour the radioactivity curve followed the optical pattern which indicates synthesis of ribosomal RNA

The results for the incorporation of *nucleosides do not confirm* the finding on ^{32}P phosphate incorporation They show however, that the pools of different precursors of RNA differ in magnitude Thus a correct interpretation of the data on the rapidly labelled HMW RNA extracted at 20° is impossible without further study of the nucleotide compositions and nucleotide sequences of the RNA fractions

Discussion of nucleic acids and granuloma age

Experiments with ^{14}C glycine The incorporation of ^{14}C glycine into DNA at different times after sponge implantation shows that most of the granuloma cells are formed during the first fourteen days After 21 days the amount of DNA decreases (Viljanto 1964) although radioactive matter is still incorporated This means that although the total number of cells is decreasing new ones are being formed at a low rate

The period of mitosis in mammalian cells has been estimated at 20 hours DNA is synthesized only during the S phase of the interphase (Lark 1963) Apparently DNA of the cells that are in the S phase at the time of removal of the granuloma are labelled These cells probably represent only a small fraction of those capable of dividing

Collagen synthesis was first observed after 7 days of granuloma development The curve (Fig 18) is similar to that of Lampiaho and Kulonen (1967) who used a different technique The high specific activity of other proteins at the time of maximal collagen synthesis suggests that they are related to the collagen synthesis On the other hand, it is important to observe that although protein synthesis is rapid on the 21st day, the incorporation of ^{14}C glycine into RNA is slower than on the 14th day Since most of the cellular RNA are ribosomal the reduction in the rate of synthesis of RNA suggests that preformed ribosomes are utilized in protein synthesis The low specific activity of non collagen proteins on the 5th day is due to extraneous protein mostly protein derived from blood plasma (Viljanto 1964)

Ribonucleic acids and granuloma age It is interesting that no changes are observed in the nucleotide composition of total RNA during the development of a granuloma though major metabolic changes are known to occur (Boucel and Noble 1963 Viljanto 1964) This is in accord with the findings of Slater and Spiegelman (1966) who studied developing *Arbacia punctulata* and concluded that early stages of embryogenesis are not accompanied by detectable differences in the major RNA components There also were no differences in the nucleotide compositions of HMW RNA extracted at 20° from proliferating and collagen synthesizing granulomas This RNA fraction consists mainly of rRNA which in turn are the major components in the total cellular RNA

The RNA extracted at 65° contain both nucleolar and chromosomal RNA The nucleolus is considered to be the source of rRNA The results suggest that the rate of synthesis of nucleolar RNA is reduced after proliferation while most of the rapidly labelled RNA extractible at 65° that are produced during the phase of collagen synthesis are chromosomal or mRNA The rapidly labelled 22S component present in HMW RNA extracted at 65° from 18 day granulomas is interesting since a rapidly labelled RNA fraction sedimenting at 22S was observed in the cytoplasmic or HMW RNA extracted at 20° only in collagen synthesizing granulomas when RNA were labelled *in vivo* with radioactive phosphate The distribution of radioactivity among the nucleotides was however different This does not necessarily mean that their nucleotide compositions are dissimilar

Harris (1961) has questioned the value of estimating nucleotide compositions of rapidly labelled RNA from the distribution of radioactive phosphate Indeed it is known that the intracellular pools of nucleoside triphosphates are not equal in size (Mandel 1964), the pool of CTP being the smallest The experiments conducted in this work also indicate that the CTP pool is smaller than the UTP pool Consequently, if much CTP is utilized in RNA synthesis pulse labelling with phosphate could lead to so high a specific activity of CTP that this is not fully compensated for by the redistribution of phosphates in the alkaline hydrolysis of RNA Since the turnover of mRNA is rapid and the nucleotides are apparently reutilized in RNA synthesis (Midgley 1963) the differences in specific activities are reduced later as seen in Table 5, where 12 hour labelling has resulted in an almost identical nucleotide composition measured either way Thus HMW RNA extracted at 65° are more rapidly synthesized than those extracted at 20°, and one can conclude that the inequalities in specific activities of precursors have diminished in the former whereas in the latter which are synthesized already earlier during the pulse the inequalities in nucleoside triphosphates have a greater effect on the distribution of ^{32}P phosphate

The peak of radioactivity in the 22S fractions of HMW RNA from 14 day and 18 day granulomas (Figs 14 and 21) and its absence from the corresponding 6 day granuloma HMW RNA preparations is of interest in respect of collagen synthesis The molecular weight of the mRNA required in the synthesis of the α chain of collagen is of the order of one million and such RNA should have a sedimentation coefficient of about 22S (Spirin 1964) However the distribution of radioactive matter in sucrose density gradients in the ^{3}H cytidine experiments was similar for granulomas of both ages Moreover 22S RNA become rapidly labelled in rat liver (Hastings *et al* 1965) and in various rat tumours (Ahonen and Kirby unpublished results) when labelled uridine is used as the RNA precursor The experiments ($^{32}PO_4$ *in vivo* and ^{3}H cytidine *in vitro*) are not however analogous since *in vivo* labelling is a pulse experiment whereas the precursor is present all the time in the other experiment *i e* the cells have a constant supply of the tracer and not only are the mRNA labelled but also other fractions The RNA component sedimenting at 22S cannot all be collagen messenger RNA because it was found to be present in the HMW RNA from 6 day granulomas

The high CMP and GMP contents of HMW RNA at the time of maximal collagen synthesis is of interest Since the LMW RNA fraction contains also rapidly labelled RNA that are hybridized to a greater extent that one

would expect for tRNA it is possible that mRNA with a high GMP and CMP content are present The molecular weight does not agree with the size of the messenger RNA for collagen mRNA apparently are single stranded structures, which are susceptible to RNAse and shear degradation Parish and Kirby (1966) have suggested that mRNA is degraded during isolation and that only the portion that is bound to the ribosome surface survives The homogenization was rather intense in the present work It has also been observed (Kumento *et al* 1966 Fig 19) that it is extremely difficult to isolate polysomes from granulation tissue It is therefore possible that part of the mRNA for collagen synthesis was degraded and was present in the sRNA fraction

In this discussion emphasis has been laid upon the mRNA for collagen synthesis It is known that collagen synthesis is associated with changes in several enzyme systems for instance the activity of the enzyme hydroxylating protocollagen increases concomitantly with collagen synthesis (Juva 1968) Increased glycolysis has been observed at the same time (Lampiaho and Kulonen 1967) These would also cause changes in mRNA as also would the little known adjustments in the nucleus that are related to the regulation of gene activities

Harris (1961) has questioned the value of estimating nucleotide compositions of rapidly labelled RNA from the distribution of radioactive phosphate. Indeed it is known that the intracellular pools of nucleoside triphosphates are not equal in size (Mandel 1964), the pool of CTP being the smallest. The experiments conducted in this work also indicate that the CTP pool is smaller than the UTP pool. Consequently, if much CTP is utilized in RNA synthesis, pulse labelling with phosphate could lead to so high a specific activity of CTP that this is not fully compensated for by the redistribution of phosphates in the alkaline hydrolysis of RNA. Since the turnover of mRNA is rapid and the nucleotides are apparently reutilized in RNA synthesis (Midgley 1963), the differences in specific activities are reduced later, as seen in Table 5, where 12 hour labelling has resulted in an almost identical nucleotide composition measured either way. Thus, HMW RNA extracted at 65° are more rapidly synthesized than those extracted at 20°, and one can conclude that the inequalities in specific activities of precursors have diminished in the former, whereas in the latter, which are synthesized already earlier during the pulse, the inequalities in nucleoside triphosphates have a greater effect on the distribution of ^{32}P phosphate.

The peak of radioactivity in the 22S fractions of HMW RNA from 14 day and 18 day granulomas (Figs. 14 and 21) and its absence from the corresponding 6 day granuloma HMW RNA preparations is of interest in respect of collagen synthesis. The molecular weight of the mRNA required in the synthesis of the α chain of collagen is of the order of one million and such RNA should have a sedimentation coefficient of about 22S (Spirin 1964). However, the distribution of radioactive matter in sucrose density gradients in the ^{3}H cytidine experiments was similar for granulomas of both ages. Moreover, 22S RNA become rapidly labelled in rat liver (Hastings *et al.* 1965) and in various rat tumours (Ahonen and Kirby, unpublished results) when labelled uridine is used as the RNA precursor. The experiments ($^{32}PO_4$ *in vivo* and ^{3}H cytidine *in vitro*) are not, however, analogous, since *in vivo* labelling is a pulse experiment, whereas the precursor is present all the time in the other experiment, i.e. the cells have a constant supply of the tracer and not only are the mRNA labelled but also other fractions. The RNA component sedimenting at 22S cannot all be collagen messenger RNA, because it was found to be present in the HMW RNA from 6 day granulomas.

The high CMP and GMP contents of HMW RNA at the time of maximal collagen synthesis is of interest. Since the LMW RNA fraction contains also rapidly labelled RNA that are hybridized to a greater extent that one

The *HMW RNA extracted at 20°* included 16S and 28S rRNA and minor components sedimenting at about 5S, 12S and 22S in sucrose density gradient ultracentrifugation (Fig 10) Pulse labelling for 2 h with radioactive phosphate resulted in a higher specific activity in *HMW RNA extracted at 65°* than in HMW RNA extracted at 20° (Table 6) Hybridization with homologous DNA suggested that both fractions contain mRNA (Table 7)

When *RNA of the subcellular fractions* from granuloma homogenates were studied it was found that ribosomes contain most of the cellular RNA whose nucleotide composition resembles that of HMW RNA extracted at 20° (Table 4) The nuclei contained HMW RNA extractable at both 20° and 65° 12S and 22S RNA were detected (besides 5S 16S and 28S rRNA) in microsomal RNA in sucrose density gradient centrifugation (Fig 11) Cytoplasmic subribosomal 40S particles obtained by zonal centrifugation (Fig 12) contained RNA sedimenting at 9S and 16S, whereas the 60S particles contained 5S 16S and 28S RNA (Fig 13)

Nucleic acids during the development of granuloma

The *rate of biosynthesis* of DNA studied by incorporation of ^{14}C glycine *in vitro* is maximal during the first week of granuloma development The synthesis of collagen is maximal during the third week Non collagenous proteins are synthesized at a high rate during the first three weeks The rate of RNA synthesis does not closely parallel the rate of synthesis of proteins (Tables 9 and 10 Fig 18)

The *extractability* of DNA decreases with increasing age of the granulation tissue whereas that of RNA remains constant

When the *nucleotide compositions* of the RNA fractions were studied it was observed that LMW RNA had higher contents of CMP and CMP during the period of maximal collagen synthesis than during cell proliferation and involution (Table 12) Differences were also observed in the nucleotide compositions of HMW RNA fractions extracted at 65° The contents of CMP and GMP were higher during the phase of rapid synthesis of collagen than during cell proliferation (Table 13) The nucleotide compositions of total RNA and HMW RNA extracted at 20° however remained constant during the granuloma development (Tables 11 and 13)

Rapidly labelled HMW RNA extracted at 20° were studied by incorporation of ^{32}P phosphate *in vivo* and ^{3}H cytidine *in vitro* Differences were observed in the distribution of ^{32}P labelled RNA in sucrose density gradients when the cell proliferation phase was compared with the phase

of collagen synthesis (Fig. 21). During the latter phase there were RNA sedimenting at 22S which were similar in molecular size to the mRNA for collagen polypeptide chain synthesis. The RNA patterns after labelling with ^{3}H cytidine were similar in this respect in both phases (Fig. 23).

When ^{32}P *phosphate labelled HMW RNA extracted at 65°* were studied during the proliferation and the phase of rapid synthesis of collagen differences were observed both in the sedimentation in density gradient ultracentrifugation and in the distribution of ^{32}P phosphate among nucleotides (Fig. 22, Table 14). This result is interpreted to indicate that synthesis of ribosomal RNA dominates during the cell proliferation phase whereas emphasis is on the production of mRNA during the phase of rapid synthesis of collagen.

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 316

DIFFERENCE IN CARDIAC ADRENERGIC INNERVATION BETWEEN HIBERNATORS AND NON-HIBERNATING MAMMALS

BY

KAI C NIELSEN and CHRISTER OWMAN

LUND 1968

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 316

FROM THE NEUROSURGICAL CLINIC A, UNIVERSITY HOSPITAL OF LUND AND THE INSTITUTE OF ANATOMY AND HISTOLOGY, UNIVERSITY OF LUND, SWEDEN

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INTRODUCTION

It has been known since the early work of WALTHER (1865) and HORVATH (1876) that the cardiac resistance to lowering of the body temperature in hibernators differs markedly from that in homeothermic (i e non hibernating) mammals For example a coordinated heart function can be maintained down to near zero in hibernating animals whereas in non hibernators the heart usually stops beating at a body temperature of about 15°C (LYMAN and CHATFIELD 1956 SARAJAS 1960 JOHANSSON 1967 1968) Although various kinds of irregular heart activity are common during hibernation ventricular fibrillation has never been observed in hibernators (JOHANSSON 1967) This cardiac failure is on the other hand the usual terminal event preceding death in homeothermic animals—including man—subjected to deep hypothermia

Several tentative explanations of this fundamental difference in cardiac resistance have been offred (see KAYSER 1957 BROOKS 1959 SARAJAS 1960 BLAIR 1964 JOHANSSON 1968) There is much evidence that reduction in temperature enhances cardiac excitability and thereby favours the development of ventricular fibrillation (BROOKS 1956 HEGNAUER and COVINO 1956 BADEER 1958 HEGNAUER and ANGELAKOS 1959 HOFFMAN 1959) Another important factor seems to be related to the hyperactivity of the sympatho adrenal system during induced hypothermia of non hibernators (BROWN and COTTEN 1956 IDE 1960 HOLOBUT 1966 NIELSEN and OWMAN 1967 NIELSEN, OWMAN and ROSENGREN 1968 BENNETT SMOOKLER and BUCKLEY 1968) it being known that catecholamines as well as stimulation of the cardiac sympathetic nerves increase the vulnerability both of the normothermic and hypothermic heart to ventricular fibrillation (BERNE 1954 HOFFMAN SIEBENS CRANEFIELD and BROOKS 1955 REISSMAN and KAPOOR 1956 HOFFMAN and SINGER 1967)

The sympatho-adrenal system plays a significant role also during the cycle of natural hibernation Available data obtained during entrance into hibernation and in the course of hibernation are controversial and often difficult to interpret because of the special technical problems encountered in such investigations The finding by SOUMALAINEN and UUSPÄÄ (1958) and UUSPÄÄ (1963 a and b) of a marked reduction in adrenal and brain noradrenaline of hedgehogs during established hibernation would indicate hyperactivity of the adrenergic system On the other hand measuring the turn-over rate of endogenously

labelled catecholamines, DRASKOCZY and LYMAN (1967) have produced evidence of suppressed adrenergic activity in the brain heart and adrenals of ground squirrels during the initial four days of hibernation However the importance of the sympathetic system during the arousal process appears to be well established (CHATFIELD and LYMAN 1950 LYMAN and O BRIEN 1963)

Though it is thus at present difficult to ascertain whether a sympatho adrenal activation occurs during corresponding phases of induced hypothermia in homeothermic mammals and of natural hibernation in hibernators it can at least be concluded that such an activation *does* occur at *some* period during the changes in body temperature Moreover since the heart also in hibernators becomes more sensitive to catecholamine influence at reduced temperatures (JOHANSSON 1967) it would seem possible that the marked difference in cardiac vulnerability between hibernators and non hibernators is related to some fundamental difference in the adrenergic sympathetic innervation of the heart

The organization of the adrenergic innervation apparatus was therefore compared in the hearts from a group of homeothermic laboratory animals (mouse rat guinea pig and cat) and a group of hibernators (bat ground squirrel and hedgehog) using a combination of fluorescence microscopic and fluorimetric methods for demonstrating catecholamines The study also included hearts from the badger because this animal is also inactive during the winter season being in a semidormant state of winter rest but differs from true hibernators in that its body temperature remains at a homeothermic level (see JOHANSSON 1957)

MATERIAL AND METHODS

Animals The studies were performed on adult animals of either sex The non hibernating laboratory animals obtained throughout the year comprised 14 albino mice (weight 20—35 g) 13 albino rats (weight 200—250 g) 13 guinea pigs (weight 500—600 g) and 15 cats (weight 2 5—4 kg)

Three active badgers (*Meles meles*) weighing between 11 and 12 5 kg were caught in southern Sweden during April—May

The hibernators were used for studies from May to October, *i e* during their active non hibernating period Eight bats (*Nyctalus noctula*) weighing about 10 g and 15 hedgehogs (*Erinaceus europaeus*) weighing 350—500 g were captured in southern Sweden Seven thirteen lined ground squirrels (*Citellus tridecemlineatus*) weighing 150—200 g were obtained from Kansas USA

Fluorimetric determinations The concentration of dopamine noradrenaline and adrenaline in the hearts was determined fluorimetrically (Table I) The animals were killed by bleeding under light ether or nembutal anesthesia and the hearts were removed and carefully freed from surrounding tissues and immediately weighed and homogenized in perchloric acid at 0—4°C The cate

cholamines were determined by the procedure of BERTLER CARLSSON ROSENGREN and WALDECK (1958) as modified by HÄGGENDAL (1963)

Cardio vascular perfusion In 2 or 3 animals from each species the vascular system of the heart was perfused with India ink to facilitate microscopic distinction between vascular and muscular adrenergic nerves The animals were anesthetized with ether the thoracic cavity was opened and a catheter connected to the perfusion apparatus was rapidly introduced cranially into the descending aorta until the tip was situated just above the aortic valves After passing the cardiac vascular system the perfusion medium (at 37°C and consisting of equal volumes of commercial India ink and 0 9 % saline with the addition of a few drops of amyl nitrite and filtered before use) was drained via the opened inferior caval vein Perfusion was continued for 1 5—3 minutes at a pressure of 150—300 mm Hg (the smaller animals) or 400 mm Hg (cats and badgers)

Fluorescence microscopy The perfused hearts and the hearts from the untreated animals not used in the fluorimetric determinations were used for fluorescence microscopic analysis of monoamines by the method of Falck and Hillarp (FALCK 1962, FALCK HILLARP THIEME and TORP 1962 CORRODI and HILLARP 1963 1964) The animals were killed by bleeding and the hearts were dissected out The hearts from the smallest animals were divided into right and left atrial portions and right and left ventricular portions Pieces from the auricular appendages both atria both ventricles and from the sino atrial and atrio ventricular node regions were taken from the hearts of the guinea pigs cats and badgers All tissue pieces were quenched to the temperature of liquid nitrogen freeze dried treated with gaseous formaldehyde at +80 C for 1 or 3 hours embedded in paraffin *in vacuo* sectioned serially (3—6 μ) and further prepared for fluorescence microscopy (FALCK and OWMAN 1965)

To identify the areas of the sino-atrial and atrio-ventricular nodes every second section from some of the preparations was stained in periodic acid Schiff and analysed in the light microscope

RESULTS

The specific formaldehyde induced fluorescence developing in the heart preparations was largely confined to nerve elements although fluorescent chromaffin cells and mast cells were also present in some regions

The adrenergic transmitter—noradrenaline—characteristically exhibits a green light under the fluorescence microscopical conditions used and can usually be visualized throughout the sympathetic neuron (see FALCK and OWMAN 1966 NORBERG 1967) The concentration of noradrenaline is highest in the terminal portion of the axon particularly in the varicosities which seem to be the specialized structures establishing the synaptic contracts with the effector cells (TLEVIN 1963 LEVER GRAHAM and SPRIGGS 1967) The preterminal part of

the nerve on the other hand has a smooth appearance and is only slightly fluorescent, in agreement with the lower concentration of the transmitter, the preterminals are therefore sometimes visible only when they form large bundles Finally the adrenergic transmitter can be visualized also in the cytoplasm of the cell body, where the concentration is of the same order of magnitude as in the preterminal part of the axon

Fluorescence microscopic analysis of the heart tissues from the four homeothermic laboratory animals—*mouse rat guinea pig and cat*—revealed principally the same arrangement of green fluorescent adrenergic nerves and chromaffin cells in the different species The fluorescence was characteristic of primary catecholamines (*cf* FALCK and OWMAN 1965) no further fluorescence developed after prolonged exposure of the tissues to formaldehyde gas which would have demonstrated structures containing a secondary catecholamine, such as adrenaline (FALCK HÄGGENDAL and OWMAN 1963) These findings agree with the fluorimetric determinations showing the presence almost exclusively of noradrenaline (Table I) Low but significant quantities of dopamine were found in the atrial portions, the implication of this finding is discussed below Adrenaline did not occur in measurable amounts in any of the species analysed

The atria were supplied with quite a rich adrenergic innervation The number of fluorescent nerve terminals was somewhat smaller in the atria of the cat (Fig 1) than in the other laboratory animals (Fig 2) The density of innervation was almost equal in the walls of both atria but there was a slight reduction in the direction from the base of the heart towards the ventricles The nerve terminals often ran in all directions not necessarily following the course of the muscle bundles (Fig 2) It was quite evident particularly in the tissues perfused with India ink that the varicose nerves were related both to the blood vessels and to the myocardium proper The axons were arranged in fairly dense plexus formations superimposed on the media of the arteries and arterioles the innervation of the venous system was less prominent Also the terminals in the myocardium ran in a characteristic autonomic ground plexus (HILLARP 1946 1959) although the typical pattern was usually evident only in sections cut in suitable planes for example parallel to the endocardial surface (Fig 3)

The terminal adrenergic innervation was particularly concentrated in the sino-atrial and atrio ventricular nodes (Fig 4) which were identified by light microscopic analysis of the sections stained in periodic acid Schiff The innervation was so dense that each of the specialized muscle cells in these regions seemed to receive at least one varicose nerve terminal

The ventricles were supplied with a moderate number of adrenergic nerve terminals and the innervation was thus usually less conspicuous than in the atrial portion of the heart The cat however was an exception having somewhat more adrenergic nerves in the ventricles than in the atria (Fig 5 and 6 to be compared with Fig 1) which agrees with the higher concentration of noradrenaline in the ventricular part (Table I) In all species the number of varicose nerves was slightly higher in the right (Fig 5) than in the left ventricle

(Fig 6) The nerve terminals in the ventricles followed the direction of the muscle bundles more strictly (Fig 5 and 6) than in the atria (see particularly Fig 2) It could be clearly established that the axons were related both to the myocardial muscle cells and to the blood vessels (Fig 5 and 6) In serial sections delicate varicose axons could often be followed for long distances when they ran close to a cardiac muscle fibre in a way suggesting a true muscular innervation The vasomotor nerves were regularly observed along vessels of calibres down that of the smallest arterioles In some of the hearts from the intravascular perfusion experiments it was even possible to see adrenergic terminals running close to a capillary

No adrenergic ganglion cells were found in any part of the hearts Intramural ganglia which could be recognized in the fluorescence microscope because of their slight unspecific autofluorescence were constantly seen in the subepicardial connective tissue near the sino atrial and atrio ventricular nodes The ganglion cells which are probably cholinergic (JACOBOWITZ 1967) sometimes also lay scattered in the right atrial wall and at the base of the aorta and pulmonary arteries Some of the ganglion cells were closely surrounded by delicate fluorescent nerve terminals in a synaptic manner Bundles of non fluorescent axons were often seen to leave the ganglion formation In addition thick bundles of smooth faintly green fluorescent nerves containing some few varicose axons entered the heart in the epicardium at the posterior surface of the atria these adrenergic nerve trunks branched into smaller fascicles to accompany blood vessels into the atria and ventricles some of them after having passed through the cardiac ganglia

Clusters of small chromaffin cells exhibiting an intense fluorescence of green to yellow green colour regularly occurred within or in the vicinity of the intramural cardiac ganglia in all species studied Isolated cells of the same type were also found in the epicardium and in the surrounding adipose tissue Usually one or two thin processes of varying length extended from the cells to run in between the non fluorescent ganglion cells or to accompany a small blood vessel or a preterminal nerve trunk for a short distance

Mast cells exhibiting a yellow fluorescence owing to their content of 5 hydroxytryptamine were present in the connective tissue particularly in the atria of the mice and rats

The number and distribution of adrenergic nerves as well as of the small strongly fluorescent catecholamine-containing cells were principally the same in the heart preparations from the *badgers* In fact the fluorescence microscopic picture in all respects closely resembled that of the *feline heart*

The principles of the cardiac adrenergic innervation in the *hedgehog* has been mentioned earlier (NIELSEN and OWMAN 1965) Both atria had an equal and fairly rich adrenergic innervation the over all amount of fluorescent nerves resembled that found in the non hibernators particularly in the atria of the guinea pig It was disclosed from the preparations with cardio-vascular perfusion of India ink that the majority of nerve terminals was directed both the

small vessels down to capillary size and to the muscle cells (Fig 7), although the axons followed the direction of the muscle fibres more strictly than in the non hibernators (Fig 7 compared to Fig 2) However, in some regions, especially near the ventricles most of the fibres were of vasomotor nature and only a few terminals could be related to the myocardial muscle cells (Fig 8) As in the homeothermic animals there was an extremely well developed innervation in both the sino atrial (Fig 9) and atrio ventricular regions

The terminal adrenergic innervation was very different in the ventricles The total number of nerves was considerably smaller than in the atria (Fig 10 and 11 to be compared with Fig 7) The left ventricle (Fig 10) had somewhat fewer fluorescent terminals than the right one (Fig 11), and the nerve fibres were exclusively found to accompany small blood vessels although most of the vascular system was devoid of adrenergic innervation (Fig 10) The arrangement in the right ventricle was similar However here at least some few isolated terminals were innervating muscle cells, as judged from their characteristic crossing over the muscle fibre without any obvious relation to blood vessels (Fig 11)

The number of fluorescent nerves in the atria of the *thirteen lined ground squirrel* was similar to that of the hedgehog—if anything somewhat larger The nerves were usually of both the vascular and muscular type and coursed quite irregularly in the atrial walls (Fig 12), although some areas seemed to have only vasomotor innervation (Fig 13) The density of innervation increased in the auricular appendages (Fig 14) and the amount of nerves was by far highest in the regions of the sino atrial and atrio ventricular nodes

The number of adrenergic nerve terminals both in the right and in the left ventricle was comparatively large in fact larger than in any of the other species in the present material (Fig 15 and 16) The arrangement of the terminals was somewhat unusual This was especially well demonstrable in the right ventricle where most of the fluorescent axons appeared as two strands on either side of almost all blood vessels of varying calibre including the smallest arterioles (Fig 15) This arrangement indicated that the vessels were in fact surrounded by a very dense plexus of nerve terminals The small vessels in the left ventricle were often accompanied by only a single varicose fibre (Fig 16) Thus the ventricles of the ground squirrel had a conspicuously well developed adrenergic nerve supply to the vascular system with the exception of scattered small regions particularly in the left ventricle, which were practically devoid of adrenergic nerves. Only some few fluorescent nerve terminals appeared not to be related to blood vessels indicating a very scantly—if any—muscular adrenergic innervation in the ventricles of the ground squirrel

The postganglionic sympathetic innervation in the heart of the *bat* differed from all other species included in the study Both the atria and the ventricles had a very poor adrenergic nerve supply (Fig 17) and most areas were completely without fluorescent nerves In regions where fluorescent terminals did occur they always ran singly accompanying a blood vessel usually for a con

siderable distance (Fig 18) The only regions with an innervation comparable to that seen in the other species were the sino atrial and atrio-ventricular nodes which received numerous adrenergic nerves probably even outnumbering the muscle cells in these special areas (Fig 19)

As in the homeothermic animals no adrenergic ganglia were found in the hearts of the hibernators However groups of non fluorescent ganglion cells were invariably recognized in the atria with the same distribution as in the non hibernators and thick bundles of non fluorescent nerves were seen to leave the ganglia. Some of the ganglion cells were closely surrounded by delicate varicose fibres exhibiting a specific catecholamine fluorescence Small branching intensely fluorescent cells emitting a yellow green light (Fig 20) were found also in the hibernators within or near the cardiac ganglia or at the base of the pulmonary artery and aorta

In the ground squirrels and the hedgehogs thick trunks of moderately fluorescent preterminal adrenergic axons entered the heart at the posterior surface of the atria (Fig 21) As expected from the scarce terminal adrenergic innervation no such bundles were found in the hearts of the bats The connective tissue in the atria of the hedgehogs contained in addition several mast cells with a green fluorescence characteristic of a primary catecholamine (Fig 21)

Table I shows that the amount of microscopically visible adrenergic nerves was in good agreement with the concentration of chemically determined noradrenaline in the heart

TABLE I *Spectrofluorimetric determinations of noradrenaline in the heart of homeothermic non hibernating laboratory animals (mouse rat guinea pig and cat) and of hibernators (bat hedgehog ground squirrel) there are no data available on the badgers The noradrenaline concentrations are expressed as Mean±S E M (number of animals within parenthesis) or as individual values*

Species	Tissue	Noradrenaline (µg/g)
Albino mouse	Whole heart	0.65±0.05 (4)
Albino rat	Atrium	1.19±0.20 (3)
	Ventricle	0.46±0.06 (3)
Guinea pig	Whole heart	1.74±0.20 (3)
Cat	Atrium	1.20±0.11 (4)
	Ventricle	1.52±0.08 (4)
Bat (*Nyctalus noctula*)	Whole heart	0.92 and 0.89
Ground squirrel (*Citellus tridecemlineatus*)	Whole heart	[illegible] and 1.03
Hedgehog (*Erinaceus europaeus*)	Whole heart	[illegible]±0.07 (3)

small vessels down to capillary size and to the muscle cells (Fig 7), although the axons followed the direction of the muscle fibres more strictly than in the non hibernators (Fig 7 compared to Fig 2) However in some regions, especially near the ventricles, most of the fibres were of vasomotor nature, and only a few terminals could be related to the myocardial muscle cells (Fig 8) As in the homeothermic animals there was an extremely well developed innervation in both the sino-atrial (Fig 9) and atrio-ventricular regions

The terminal adrenergic innervation was very different in the ventricles The total number of nerves was considerably smaller than in the atria (Fig 10 and 11 to be compared with Fig 7) The left ventricle (Fig 10) had somewhat fewer fluorescent terminals than the right one (Fig 11) and the nerve fibres were exclusively found to accompany small blood vessels although most of the vascular system was devoid of adrenergic innervation (Fig 10) The arrangement in the right ventricle was similar However here at least some few isolated terminals were innervating muscle cells as judged from their characteristic crossing over the muscle fibre without any obvious relation to blood vessels (Fig 11)

The number of fluorescent nerves in the atria of the *thirteen lined ground squirrel* was similar to that of the hedgehog—if anything somewhat larger The nerves were usually of both the vascular and muscular type and coursed quite irregularly in the atrial walls (Fig 12), although some areas seemed to have only vasomotor innervation (Fig 13) The density of innervation increased in the auricular appendages (Fig 14) and the amount of nerves was by far highest in the regions of the sino-atrial and atrio ventricular nodes

The number of adrenergic nerve terminals both in the right and in the left ventricle was comparatively large in fact larger than in any of the other species in the present material (Fig 15 and 16) The arrangement of the terminals was somewhat unusual This was especially well demonstrable in the right ventricle where most of the fluorescent axons appeared as two strands on either side of almost all blood vessels of varying calibre including the smallest arterioles (Fig 15) This arrangement indicated that the vessels were in fact surrounded by a very dense plexus of nerve terminals The small vessels in the left ventricle were often accompanied by only a single varicose fibre (Fig 16) Thus, the ventricles of the ground squirrel had a conspicuously well developed adrenergic nerve supply to the vascular system with the exception of scattered small regions particularly in the left ventricle which were practically devoid of adrenergic nerves Only some few fluorescent nerve terminals appeared not to be related to blood vessels indicating a very scantly—if any—muscular adrenergic innervation in the ventricles of the ground squirrel

The postganglionic sympathetic innervation in the heart of the *bat* differed from all other species included in the study Both the atria and the ventricles had a very poor adrenergic nerve supply (Fig 17) and most areas were completely without fluorescent nerves In regions where fluorescent terminals did occur they always ran singly accompanying a blood vessel usually for a con

In the discussion on differences in cardiac resistance with special reference to the incidence of ventricular fibrillation at reduced temperature it seems important to point out that the hibernators and the non hibernators resemble one another regarding the adrenergic innervation of the atria as well as of the sino atrial and atrio ventricular nodes

The presence in all species of the small intensely fluorescent cells in the atria particularly those near or within the non adrenergic cardiac ganglia may be of special significance in cardiac function For the sake of simplicity these cells have been named 'chromaffin because they resemble adrenal medullary chromaffin cells Strictly speaking however the term chromaffin is inappropriate because the cardiac cells are usually not stained with the chromaffin reaction (JACOBOWITZ 1967) The conspicuously yellowish appearance of the cells in the fluorescence microscope is probably due to their very high catecholamine concentration which may give rise to secondary reactions with formaldehyde resulting in the formation of a new yellowish fluorescent product (CASPERSSON HILLARP and RITZEN 1965) Or it may be explained by the fact that the maximum sensitivity of the human eye is shifted towards longer wavelengths when the intensity of the light increases (WRIGHT 1944) Microspectrograpic analysis has revealed that the catecholamine stored in these cells is dopamine (EHINGER FALCK PERSSON and SPORRONG 1968) It is thus most probable that the dopamine measured in the heart particularly in the atria is present only in chromaffin cells rather than in nerve terminals of for example the sino atrial node region (EHINGER FALCK PERSSON and SPORRONG 1968 *cf* ANGELAKOS FUXE and TORCHIANA 1963) There is strong reason to believe that the cardiac chromaffin cells receive a cholinergic innervation (JACOBOWITZ 1967) and the presence of the cellular processes—which sometimes even have a varicosed appearance resembling nerve terminals (OWMAN and SJOBERG 1966 NIELSEN 1968)—within the cardiac ganglia and nerve trunks may indicate that catecholamines can be released from the cells to influence the ganglionic transmission within the heart perhaps as an intrinsic feedback mechanism The chromaffin cells might thus act to supplement the adrenergic innervation present in the cardiac ganglia in a postulated inhibition of ganglionic transmission (see JACOBOWITZ 1967)

As expected the cardiac adrenergic innervation of the badgers was equal to that of the non hibernating laboratory animals Although badgers resemble hibernators in their immobility and loss of weight during their winter sleep their body temperature remains at a homeothermic level in contrast to that of truly hibernating animals (JOHANSSON 1957) In agreement with this the cardiac reaction to induced hypothermia in badgers is quite different from that of the hibernating or hypothermic hedgehog but corresponds closely to the reaction of the dog (JOHANSSON 1957)

The finding of a difference in the organization of the adrenergic innervation in the heart ventricles of hibernators and homeothermic mammals have been the basis for a series of investigations on the significance of adrenergic mecha

nisms in the production of ventricular fibrillation during induced hypothermia in cats The results of these experiments have shown that such mechanisms notably at the level of the heart play a major role in the development of this serious complication of induced hypothermia and that hypothermic ventricular fibrillation can be effectively prevented by pharmacological and surgical interference with various steps involved in the sympathetic influence on the adrenergic receptors (NIELSEN and OWMAN 1965, 1966 1967, 1968 a c)

SUMMARY

It is well known that the cardiac resistance to reduction in temperature is markedly different in hibernators and non hibernating mammals For example ventricular fibrillation never occurs in hibernating animals whereas this the usual terminal event preceding death in non hibernators subjected to deep hypothermia The reason for this difference is not understood

The present study was undertaken to investigate whether the difference in cardiac resistance to lowering of the temperature could be explained by some difference in the terminal adrenergic innervation apparatus of the heart The adrenergic transmitter was demonstrated by fluorescence microscopy of formaldehyde treated preparations and by spectrofluorimetric analysis

The number and distribution of adrenergic nerve terminals in the atria of the hibernators (bat hedgehog and thirteen lined ground squirrel) resembled that of the non hibernating animals (mouse rat guinea pig cat and also the badger) Both groups of animals possessed a very dense adrenergic innervation of the sino-atrial and atrio ventricular nodes Intensely fluorescent catecholamine-containing chromaffin cells regularly occurred in the atria of all animals No adrenergic ganglia could be demonstrated

The ventricles of the non hibernating animals were well supplied with adrenergic nerve terminals running both to the vascular system and to the myocardial muscle cells This contrasted sharply with the distribution of fluorescent nerves in the ventricles of the hibernators only scattered terminals were found in the bat and those present invariably accompanied blood vessels The ventricles of the ground squirrel and hedgehog received a fairly large number of adrenergic nerves but they ran almost exclusively in relation to the vascular system

The finding demonstrate a fundamental difference in the adrenergic nerve supply to the myocardial muscle fibres in the ventricles of hibernators compared with non hibernating mammals the almost complete lack of adrenergic innervation in the myocardium of hibernators may be one explanation for the higher resistance of these animals to lowering of the body temperature

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FLUORESCENCE PHOTOMICROGRAPHS

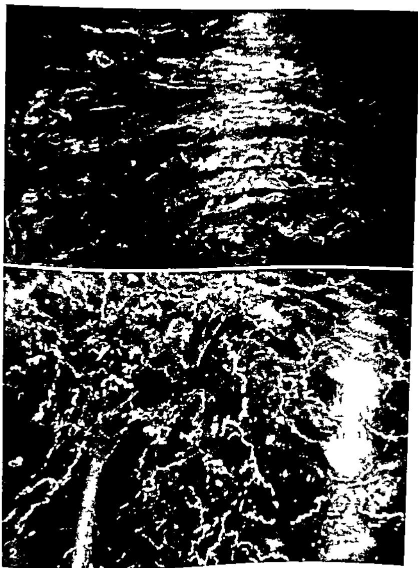


Fig 1 Cat atrium Moderate number of adrenergic nerve terminals running both to the blood vessels and to the myocardial cell 100X

Fig 2 Mouse atrium Considerable number of varicose adrenergic nerves coursing in all directions in the atrial wall 180X

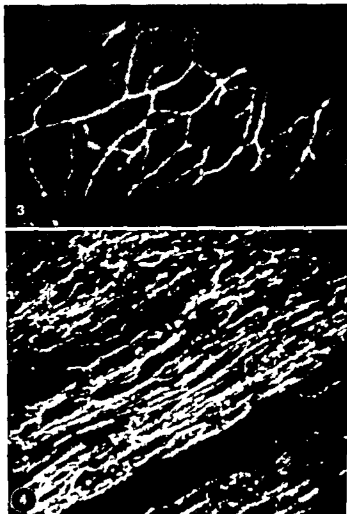
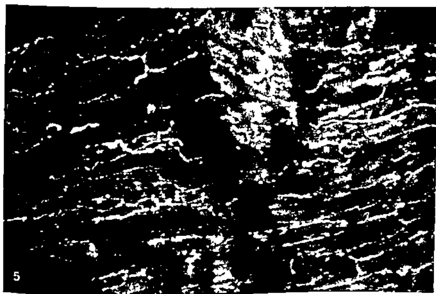


Fig 3 Guinea pig atrium The section is cut parallel to the endocardial surface and demonstrates the varicosed fluorescent nerve terminals running in a typical autonomic ground plexus that innervates the myocardium 300X

Fig 4 Guinea pig atrioventricular node Extremely rich supply of fluorescent terminals it can be assumed that each cell in the node receives at least on adrenergic axon 90X



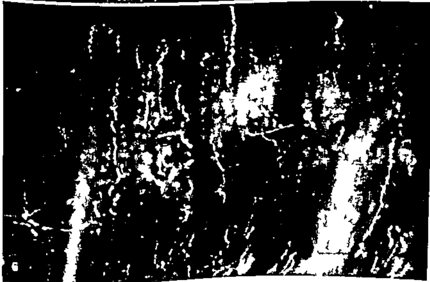


Fig 5 Cat right ventricle Rich distribution of varicosed adrenergic nerve terminals following the direction of the muscle fibres and innervating both vessels and the myocardium proper 140X

Fig 6 Cat left ventricle Fewer fluorescent axons but with the same distribution as in the right ventricle Small autofluorescent granules in the myocardial cells 160X

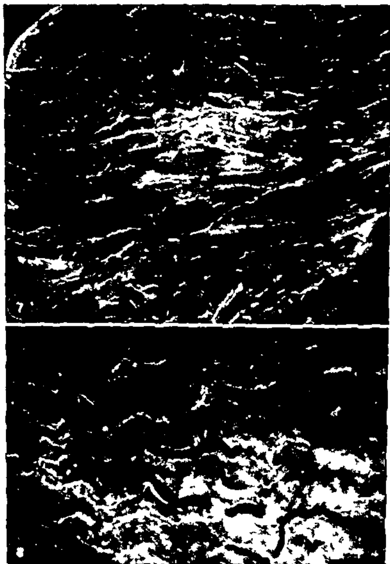


Fig 7 Hedgehog atrium India ink perfusion The black blood vessels are well visible The fluorescent nerves are seen to be related both to vessels and to myo ardial fibres whose g neral di tion they follow 90X

Fig 8 He lg h g atrium India ink perfusion In this region near the ventricles the fluorescent er als are seen to follow blood vessels almost exclusively 140X

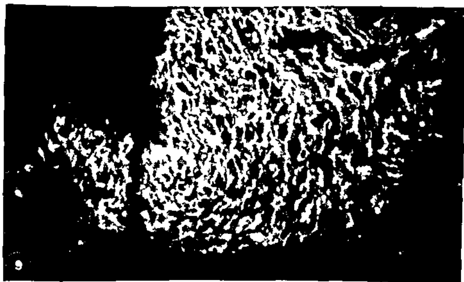
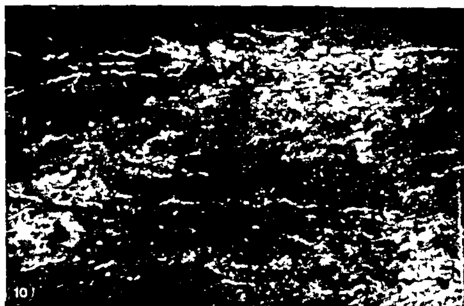


Fig 9 Hedgehog sino atrial node Most of the cells in the node are cut transversally and they are seen to be densely surrounded by fluorescent terminals 250X



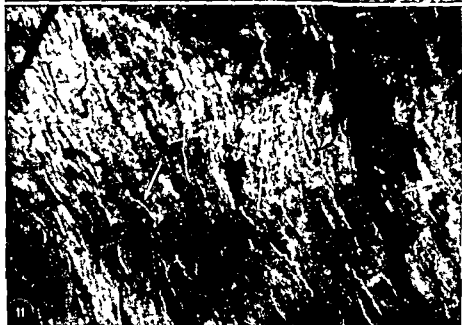


Fig 10 Hedgehog left ventricle India ink perfusion Most of the vascular system is devoid of fluorescent axons those present are always seen to accompany a vessel no nerve being related to the myocardial muscle fibres 100×

Fig 11 Hedgehog right ventricle India ink perfusion Somewhat larger number of nerves than in the left ventricle The bulk of fluorescent nerves accompany vessels although some few fibres (arrows) seem to be related to the myocardial muscle cells 100×

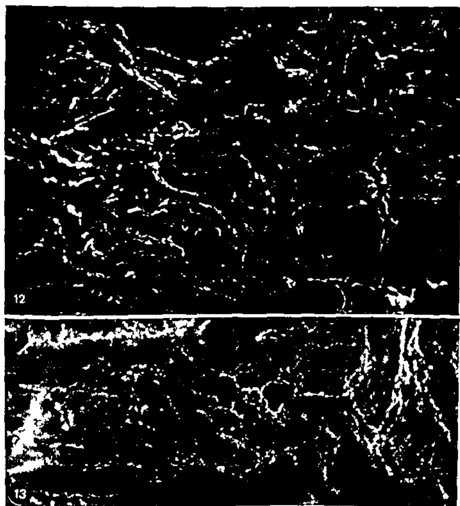


Fig 12 Ground squirrel atrium The atrial wall has a rich supply of adrenergic axons running in all directions both to vessels and to the muscle fibres 150X

Fig 13 Ground squirrel atrium In this area the vessels are seen to be accompanied by two strands of varicose nerves running on either side of the wall No nerves are found in the myocardium proper 100X

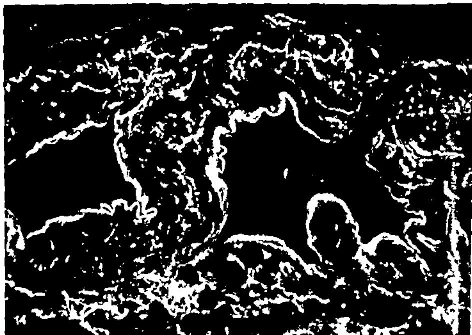


Fig. 14 Ground squirrel auricular appendage Dense adrenergic innervation in the muscular wall Autofluorescence in the lumen Border 125X





Fig 15 Ground squirrel right ventricle Large number of adrenergic nerve terminals almost exclusively accompanying blood ves els in the form of two dense strands on either side of the walls 150X

Fig 16 Ground squirrel left entricle India ink perfi ion The vascular system is well supplied ith adrenergi nerves single arico e term l clo ely accompanying almost all of the small ves els Note that no terminal can seen in the myocardium proper without relation to ve sels 150X



Fig 17 Bat right ventricle India ink perfusion The myocardial fibres are near transversally sectioned as seen from the appearance of the vascular system which is black in the figure No fluorescent nerves occur in the myocardium proper Only three groups of fluorescent nerves are visible all related to large blood vessels (arrows) 140×

Fig 18 Bat left ventricle India ink perfusion The section is cut parallel to the direction of the myocardial fibres which receive no fluorescent nerves Those few adrenergic nerve terminals present (arrows) are seen to accompany blood vessels sometimes for a considerable distance 200×



Fig 19 Bat sino-atrial node Extremely rich supply of adrenergic nerve terminals in the node which is cut mainly transversally to the left and longitudinally to the right Note that surrounding myocardial tissue is without fluorescent nerves 100×

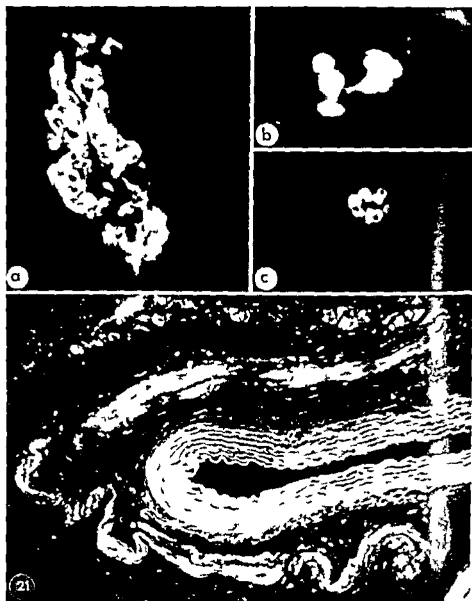


Fig 20 Groups of intensely fluorescent chromaffin cells sometimes with a well visible process in the right atrium of the hedgehog (a) ground squirrel (b) and bat (c) 175×

Fig 21 Hedgehog tissue taken at the base of the pulmonary artery The elastic tissue in the transversally sectioned arterial wall has an intense unspecific autofluorescence Thick bundle of smooth preterminal fluorescent nerves runs close to the vessel Green fluorescent mast cells are scattered in the tissue 60×

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 317

TRANSFORMATIONS OF RAT SKIN COLLAGEN

WITH SPECIAL REFERENCE TO THE AGEING PROCESS

TURKU 1968

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 31

FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF TURKU
TURKU, FINLAND

TRANSFORMATIONS OF RAT SKIN COLLAGEN

WITH SPECIAL REFERENCE TO THE AGEING PROCESS

BY
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TURKU 1968

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Turku Kirjapaino Polytypos 1968

CONTENTS

ABBREVIATIONS

Hyp	hydroxyproline
TC	tropocollagen
NSC	0.45 *M* NaCl soluble collagen
ASC	0.5 *M* acetic acid soluble collagen
ISC	collagen insoluble in 0.5 *M* acetic acid at 4 C (unless defined otherwise)
ISC	fraction of insoluble collagen dissolved by 0.5 *M* acetic acid at 40
ISC_n	$ISC-ISC_{40}$
Aldehyde/TC	moles of acetaldehyde equivalent per mole of tropocollagen

INTRODUCTION

The association of tropocollagen monomers to ordered aggregates and the increase in the stability of these polymeric structures with age are fascinating and unique features in protein metabolism.

Tropocollagen once secreted into the extracellular space forms various supermolecular structures depending on the tissue in question. The reactions of collagen with calcium and/or phosphate are the first steps in the calcification of bone. In soft connective tissue a macromolecular network is formed by the interaction of the collagen molecules with each other and probably also with other connective tissue components, e.g. glycosaminoglycans. The formation of intra and intermolecular linkages is believed to be the main reason for the increase in the stability of the extracellular matrix with age. In addition, as will be shown below, calcium ions may play some part in the stabilization of collagen in soft connective tissue. The biological ageing of collagenous structures is correlated with many metabolic and structural alterations such as a retardation of collagen synthesis, a decrease in the proportion of extractable collagen, an increase in the rigidity of the tissue and an increase in the contractile force which develops in the tissue.

One of the classic hypotheses of ageing is that changes in the 'inert' materials of the body (collagen and other intercellular substances) limit the life span of the whole organism (Comfort 1965). Numerous reviews (Strehler 1962, Comfort 1965, Verzar 1963, Curtis 1966, Sinex 1968, Bjorksten 1968) confirm that the hypothesis is supported by experimental data. It appears that changes in collagenous structures, probably resulting from the deterioration of connective tissue cells, contribute to some symptoms of old age by damaging blood vessels, wrinkling the skin and lowering the rate of wound healing. It is possible that similar mechanisms contribute also to the ageing of other macromolecules which thus inactivate important molecular elements.

Despite the growing pace of investigation the mechanism of formation of insoluble collagen in the body is obscure as is also the alteration of insoluble collagen with advancing age and in pathological conditions.

The purpose of the present study was to obtain information about the formation of insoluble collagen with special reference to the ageing process and to factors involved in the development of supermolecular networks of collagen. It was hoped that the results would contribute to a better understanding and a more precise definition of the problem.

When this study was begun it was understood that collagen will reveal its secrets only with reluctance and the problem of maturation and ageing of collagen may attract many others to undertake simultaneous research projects. However, the long tradition of research in the field of connective tissue chemistry and the inspiring atmosphere of the department encouraged the author to undertake the present study.

REVIEW OF THE LITERATURE

Formation of insoluble collagen *in vivo* and *in vitro*

The fraction extracted from fresh connective tissue by cold sodium chloride solution is generally believed to represent the earliest form of collagen which is progressively converted into insoluble fibres (Harkness *et al* 1954 Jackson 1957, Gross 1958 1964 Green and Lowther 1959, Jackson and Bentley 1960, Kuhn *et al* 1964a, b) The major part of the neutral salt soluble collagen is later converted into acid soluble and insoluble form This process is so slow that it takes several weeks (Tsurufuji and Nakagawa 1967) The acid soluble collagen is supposed to result from the formation of cross links between α components in the tropocollagen molecule The formation of insoluble collagen may involve both the formation of cross links between tropocollagen molecules and reactions of tropocollagen molecules with other connective tissue components (Piez *et al* 1961 Bornstein and Piez 1964 Gallop 1964 Heikkinen *et al* 1964 Heikkinen and Kulonen 1964 Kühn *et al* 1964a b Robertson 1964 Veis and Anesey 1965)

Maturation of connective tissue is accompanied by a decrease in the content of hyaluronic acid and a relative increase in the content of sulphated glycosaminoglycans especially chondroitin sulphuric acids (see reviews by Sinex 1968 and Lehtonen 1968) Hyaluronic acid is believed to reduce the rate of aggregation of tropocollagen molecules Older connective tissue may run out of cells capable of maintaining its ground substance matrix and thus may permit the condensation of tropocollagen molecules into large aggregates (Sinex 1968) Also mucoproteins are supposed to play an important role in determining the structural stability of insoluble collagen (see review by Banga 1966) Complete dispersion of insoluble collagen in 0 1 *M* acetic acid is achieved after treatment with α amylase from *B subtilis* (Nishihara 1963 Steven 1964 1966) This points to the importance of glyco or mucoproteins in the formation of insoluble collagen

Recently, various models have been proposed for the products of interaction of glycosaminoglycans mucoproteins and collagen The existence of a chondroitin sulphate protein complex was proposed by Mathews (1965) This complex consists of a protein core to which chondroitin sulphate molecules are attached The protein core is aligned in parallel with the collagen fibril This model has been extended to include the mucoproteins in connective tissue (Jackson and Bentley 1968) The model implies that collagen aggregates must interact with mucoproteins to attain a level of fibrillar organization The fibrils are then converted into fibres by the formation of chondroitin sulphate protein complexes

The metabolic relationships between the fractions of collagen that differ in solubility is not clear. In their work with carrageenin granuloma Jackson and Bentley (1960) showed that radioactive matter was not transferred directly from the fraction soluble in 0.14 M sodium chloride to the citrate soluble fraction but was transferred from collagen extracted by dilute neutral salt solution to collagen extracted by neutral salt solution of higher ionic strength. Thus it would appear that the longer the time that has elapsed since a collagen molecule was synthesized the more firmly will it be bound into a collagen aggregate. This will be a continuous process and so at any given time there will be a continuous spectrum of collagen aggregates of varying degrees of cross linking. However, the collagen of rat skin that is soluble in acid buffers or in dilute acid seems not to be an active precursor of insoluble collagen (Robertson 1964, Tsurufuji and Ogata 1965, Tsurufuji and Nakagawa 1966). The rate of formation of insoluble collagen varies also with the age of the animal (Heikkinen and Kulonen 1969) and has been suggested to be tissue specific (Veis 1967).

Reconstitution of collagen fibres from solutions by different agents and the regeneration of the collagen structure on cooling denatured collagen below the denaturation temperature have been discussed in relation to cross linking and the formation of insoluble collagen (Cross and Kirk 1958, Gross 1958, 1961, 1964, Kuhn *et al.* 1959, Lowther 1963, Majamaa and Kulonen 1964, Bello and Bello 1966, Bugby 1966, Bailey 1968).

Electron microscope studies (Randall *et al.* 1955, 1955, Schmitt *et al.* 1953) have shown that collagen fibres precipitated from solution are identical with native fibre with the 640 Å periodicity. If precipitation is performed with polyanions such as chondroitin sulphate fibrils with a repeat period of 2000–3000 Å (fibrous long spacing FLS) are formed (Highberger *et al.* 1951). Addition of ATP to an acid collagen solution produces the so-called segment long spacing (SLS) form of collagen (Schmitt *et al.* 1953). Most of the fibres reconstituted from tropocollagen *in vitro* will dissolve in salt solution and acid buffers and lack the stability of native fibres (see review by Wood 1964). Numerous studies have dealt with the precipitation of collagen fibrils from solution by different glycosaminoglycans (see review by Jackson and Bentley 1968) but at this stage it is not possible to draw any final conclusion about the relationship between the biological processes and the *in vitro* processes.

Incubation of collagen solutions near pH 7 at 37° leads to the formation of collagen gel (Cross 1958). Subsequent dissolution of the gel on cooling depends on the length of the incubation period. If incubation is carried out for a longer period (days and weeks) the stability of the gel increases and it becomes finally insoluble even in acid buffer (Cross 1958). Fessler (1960) demonstrated that highly purified neutral salt-soluble collagen varies in its ability to form fibres on warming to 37° and to redissolve on cooling to 4°. The chemical basis of the variation in the precipitation and redissolution is not yet understood.

The results of Cross (1958, 1961) led him to suggest that the loss of solubility is not due to interaction of collagen with non-collagenous components but to the formation of more stable sterically organized aggregates stabilized by increased binding of the tropocollagen molecules as a result of Brownian movement.

Recently Bello and Bello (1967) suggested spontaneous cross-linking of collagen. Incubation of aqueous collagen gel at 37° led to the formation of insoluble collagen which was considered to be covalently cross linked. The addition of histidine, arginine, lysine or EDTA and removal of oxygen retarded the cross linking processes. The

results of Tsurufuji and Ogata (1966) and Chvapil (1968) suggest that aerobic cellular activities are necessary for the increase in the stability of the collagen fibre Heikkinen and Kulonen (1967) and Heikkinen et al (1968) using other technique than these authors showed that the formation of insoluble collagen in vitro was not dependent on aerobic cellular activities Incubation of granuloma homogenates in vitro reduced the solubility of soluble collagen (Majamiemi and Kulonen 1964) but collagen which had been added either as a solution or as a suspension partly dissolved during incubation in Krebs Ringer phosphate at 3

Both spontaneous and metabolically controlled formation of insoluble collagen in vitro have been suggested At this stage it is not possible to correlate the results with the biological situation

Molecular anatomy of the interchain linkages of collagen

It has been established with certainty that interchain linkages occur in collagen These interchain cross links determine for instance the hydrothermal stability and solubility of collagen (tertiary and quaternary structures) The necessity for assuming the existence of cross links in collagen has come from e g collagen gelatin transformation (see review by von Hippel 1967) The collagen gelatin transformation is usually defined as a phase transition from a helical structure to a random coil This transition may be detected e g by determining the denaturation temperature T_D (the temperature at which the specific viscosity falls to 50 % of its initial value Doty and Nishihara 1958) or by determining the temperature of thermal shrinkage T_S The value of T_D of mammalian collagens lie between 36 and 39 and the values of T_S between 60 and 70 (Pikkarainen 1968) Native collagen swells and its T_S and T_D decrease when the pH of the solution is changed from neutral (Gustavson 1956 Crosby and Stainsby 1962) It has been postulated by Rigby (196) that the swelling of collagen is reversible below a characteristic temperature T_T and a second irreversible swelling takes place at or above T_T T_T was found to coincide with T_D. Partial reversion of gelatin to the native state at a temperature below T_D can always be effected but total reversion is only achieved in special circumstances (see review by von Hippel 196) This kind of transition has also been discussed with reference to interchain linkages During renaturation the changes in viscosity optical rotation volume susceptibility to proteolysis and other properties caused by denaturation are reversed

The nature of the interchain linkages will be discussed in the following Research in this field has proceeded in a number of different ways Several suggestions about the nature of the interchain linkages have been made and all of them have gained some experimental support

Electrostatic bonds

The side chains of the amino acids of the protein may be nonpolar groups polar groups not charged in neutral solution and either positively or negatively charged ionic groups

The positively charged groups are represented by the e amino group of lysin

the guanidium group and free α amino group. Carboxyl groups are negatively charged groups The charged groups of collagen may form electrovalent links and metal mediated links It has been postulated (Kern 1960) that approximately 95 % of the ionic sites in steer hide collagen exist in some form of internal electrostatic compensation The strengths of these links largely depend on the distance between the charged groups and the links are easily broken by acids and bases The charge profile of collagen determines its molecular orientation e g the quarter staggering of tropocollagen molecules It has been suggested (Cross 1961) that the increase in the stability of collagen with age is due to a gradual packing of collagen molecules which results from the formation of electrostatic bonds between the molecules and leads to a more crystalline structure

Hydrophobic bonds

The association of nonpolar molecules in aqueous solution is termed non polar or hydrophobic bond formation It has been suggested that the formation of nonpolar bonds is one of the most important factors influencing the high order structures of proteins (Mahler and Cordes 1967) The primary structure of collagen contains sections that are composed mainly of neutral amino acids and sections composed mainly of polar amino acids (see review by Ramachandran 1967) In addition to the mutual orientation of collagen molecules hydrophobic bond formation with other extra cellular components e g lipids may influence the matrix properties

Hydrogen bonds

According to available information both the thermal shrinkage of the collagen fibre and the denaturation of the collagen molecule in solution results from the rupture of hydrogen bonds (see review by Bailey 1968) The abundance and stability of these bonds determine the denaturation temperature Different numbers of hydrogen bonds have been proposed for every three amino acids of the peptide chain depending on the model of collagen (see review by von Hippel 1967) The presence of glycine as every third residue make possible the formation of hydrogen bonds which stabilize the triple helix The positions of the pyrrolidine residues in the tripeptides apparently determine the stability of the triple helix by restricting bond rotation and allowing the formation of interchain bonds in nonpolar regions It has been demonstrated that the presence of interchain covalent links has little effect on T_D (Veis and Drake 1963) Also water molecules may form interchain hydrogen bonds (Berendsen and Migchelsen 1966) Gradual loss of ground substance with advancing age and consequent dehydration of the tissue may be of primary importance in promoting greater interaction of the collagen molecules

Ester bonds

Gustavson (1956) suggested that ester linkages occur in gelatin obtained from insoluble collagen Gallop *et al* (1959) found hydroxylamine sensitive linkages in acid soluble carp swim bladder collagen A bond between a carboxyl group of one peptide chain and the ε amino group of lysine in another peptide chain has been proposed by Lewy *et al* (1960) *They found that about 30 % of the* ε amino groups *of lysine*

residues do not react with nitrosyl chloride Hormann has however shown that all the ε amino groups react when the reaction is performed in denaturing conditions (e g in the presence of NaClO) Franzblau *et al* (1961) have also found ester cross links in collagen which are sensitive to hydroxylamine About six intramolecular ester bonds per 100 000 molecular weight units were present

Blumenfield *et al* (1962) found evidence that the cross links of denatured collagen disrupted by treatment with hydrazine involved the carboxyl groups of aspartic acid residues They suggested further that the cross links involving aspartyl residues occurred in pairs separated by no more than seven amino acids A Lossen rearrangement of hydroxylamine treated gelatin and lithium borohydride reduction of untreated gelatin provided the evidence that half of the bonds were formed by β carboxyl groups and half by α-carboxyl groups These investigations are discussed by Gallop (1964) The participation of carbohydrates in the formation of ester bonds is discussed later in this chapter

Linkages involving carbohydrates

The investigations of Grassmann and Hormann (1960—1963) stressed the significance of the collagen carbohydrate bonds Even after extensive purification collagen contains about 0 5 % hexose Hormann found (1962) that the addition of hydroxylamine after disruption of hydrogen bonds makes collagen completely soluble and that periodate destroys the hexose bridges in collagen and makes thermally contracted collagen completely soluble The possibility was discussed that the cross links of collagen are hexose bridges According to Hörmann (1960 1962) the reducing group of a hexose residue should react O glycosidically with a hydroxyl group of one peptide chain and one of its hydroxyl groups should form an ester bond with a carboxyl group of an adjacent peptide chain

Butler and Cunningham (1966) suggested that a disaccharide composed of glucose and galactose is linked O glycosidically to the hydroxyl group of a hydroxylysine residue in a special peptide region of the collagen molecule and that the disaccharide may form cross links in collagen during maturation

The importance of hexoses in the formation of cross links has also been postulated by Bensusan *et al* (1966) who suggested that hexoses form N glycosidic linkages with the ε amino groups of lysine and/or hydroxylysine residues in collagen These glycosides could then form Schiff's base and react further with the imidazole rings of histidine residues in an adjacent chain

Linkages involving aldehydes

Recently attention has been focused upon the presence of aldehyde like groups in collagen (Pajkind *et al* 1964) Free carbonyl groups were found in gelatins by Landucci already in 1954 and later by Levene (1962) who showed that there are fewer carbonyl groups in lathyritic collagen than in normal collagen and explained thus the decreased cross linking (Martin *et al* 1961 Nikkari and Kulonen 1962)

One type of intramolecular cross link may be formed in the β components of collagen after the conversion of lysine residues in the chain to aldehyde derivatives (Bornstein *et al* 1966) Lathyritic collagen was found to lack these carbonyl groups Tanzer *et al* (1966) found that intermolecular cross linking could be inhibited with

thio micarbazide in vivo but that there resulted no deficiency of intramolecular cross links, this suggests that the cross links are not necessarily interdependent Rojkind *et al* (1964, 1966) isolated a peptide with aldehydic group from ichthyocol collagen and Bornstein *et al* (1966) a similar peptide from rat skin collagen The observations suggest that aldehydes are important in the cross linking and maturation of collagen In later experiment Rojkind and Juárez (1966) found that lathyrism caused a 50 % decrease in the carbonyl groups of the α1 chains and suggested that aldehyde formation in the α1 chain may be the major rate limiting step in the overall process of cross linking and maturation Kang *et al* (1966) observed that each of the α components of rat skin collagen contains one aldehyde function and the results of Rojkind and Juarez (1966) indicate that in lathyrism only one of the α chains has less than one aldehyde group on average

The role of telopeptides

One of the cross linking functions has recently been attributed to telopeptides which were introduced into the chemistry of collagen by Rubin *et al* (1963) From the triple helix body of native tropocollagen protrude terminally or laterally peptides having a composition different from that of the triple helix itself (Hodge *et al* 1960) These peptides are removed by proteases and with their removal intramolecular inter chain bonds are supposed to be broken It was further (Drake *et al* 1966) verified that pronase converted β and γ components to α components with concomitant release of 1—5 % of dialysable peptides The action of pepsin and pronase on native collagen solutions resulted in the release of dialysable peptide devoid of hydroxyproline and hydroxylysine residues, but rich in tyrosine aspartic acid and glutamic acid residue (Rubin *et al* 1965 Bump *et al* 1967) Because the formation of fibrous aggregates of tropocollagen was also considerably inhibited it was concluded that one or more of the peptides must be located in peripheral regions of the tropocollagen molecule

Telopeptides may play a part in the aggregation of tropocollagen to microfibril forms in which the aggregation is more end to end than side to side (Veis 1967)

Transformations of skin collagen with age

Changes in solubility

There are numerous reviews on the solubility of collagen in relation to the formation of cross links and biological ageing (Harkness 1961 Wood 1964 Grassman 1965 Harding 1965 Veis 1965 Langa 1966 Piez 1967, Bailey 1968) The collagen content of mammalian skin varies with the species and age (Table I) The accumulation of collagen in skin with age is accompanied by a decrease in the proportion of extractable collagen (Table II) Collagen extractable by neutral buffers essentially disappears during ageing It has been lately shown however that the greater part of the adult rat skin collagen dissolves in native form in salt and dilute acetic acid solutions (Heikkinen and Kulonen 196[illegible] Tanzer, Fujii and Nakagawa 196[illegible] Heikkinen and Kulonen 1968) It is thus apparent that the present definition of soluble collagens is not satisfactory The various extractants [illegible] used by different authors are responsible for the contradictory results on [illegible] of soluble collagens

Table I Collagen contents and variation of extractable collagen content with age in skins of man rabbit and rat

The figures are the collagen content and relative proportions of extractable collagen between the indicated ages Systematic studies of the effect of age are analysed further in Table II

Source	Age range	Per cent collagen in		Per cent collagen extracted by			Reference
		fresh skin	dry skin	neutral salt solutions	acid buffer solutions	acetic acid solutions	
Man	Newborn–80 years				0.0[illegible]–0.18		Bakerman (1964)
	Newborn			0.54		6.9	Bornstein and Piez (1964)
	6 months–80 years		[illegible]–[illegible]8				Clausen (1962)
	[illegible]–60 years		69.5–95.0			1.[illegible]–3.[illegible]	Harris and Sjoerdsma (1966)
	Adult	14–16.4		0.08	0.09–0.[illegible]3		Korting *et al* (1964)
	[illegible]– years		39.[illegible]–47.6				Sobel *et al* (1958)
Rabbit	[illegible] weeks–9 months	10–16.5		6.1–13	3.0–4.1		Nimni *et al* (1966)
Rat	Embryo–1 year	11–[illegible]3.4	6.2–18	1.0–9.5	3.1–13.0		Cadavid *et al* (1963)
	1 month			[illegible]0.9		29.4	Heikkinen and Kulonen (1966)
	Adult	15.[illegible]–19.2		4.[illegible]–7.6	4.0–6.4		Holzmann *et al* (1964)
	Adult	18.8–19.7		0.10	14		Kuhn *et al* (1964a b)
	Newborn–1 year	10.3–17.8		4.8–[illegible]4.9	[illegible]5–8.6		Mills and Bavetta (1966)
	10–60 days			[illegible]–59.8		1[illegible]9–37.1	Tsurufuji and Nagakawa (196[illegible])
	1 day–[illegible] years			0.3–4.9			Wirtschafter and Bentley (1962)

Calculated from the original data (mg extractable collagen/g normal skin) using the data of Mills and Bavetta (1966) for total collagen content of rat skin (per cent of wet weight)

Table II The variation of the solubility and content of skin collagen with age

Collagen contents (per cent of wet weight) and relative proportions (parts per hundred) of collagen fractions in skins of rats and rabbits of different ages are given

Animal and collagen fraction	Age of animal											Reference
	Newborn	7 days	11 days	14 days	21 days	30 days	60 days	90 days	6 months	9 months	12 months	
Rat												
0 4 M NaCl (pH 7 4)	4 9	4 2		5 3		4 5	1 1				0 3	Wirtschafter and Bently (1962)
Rat												
Collagen content	1 1		1 9		4 2		15 7		20 0		23 4	Cadavid *et al* (1963)
0 5*M* NaCl soluble	6 8		9 5		9 0		5 0		1 0		1 0	
0 2*M* citrate soluble (pH 3 5)	3 1		5 5		13 0		3 0		5 0		5 5	
Rat												
Collagen content	1 0	1 9		_3		10 7	10 4				2 0	Mills and Bavetta (1966)
0 15*M* NaCl soluble	1_5	14 3		1_4		4 9	5 6				2 8	
0 5*M* NaCl soluble	1_6	21 2		39 5		_2 4	1_3					
0 5*M* citrate soluble (pH 3 6)	3 9	4 9		4 8		2 5	8 6				4 9	
0 3*M* TCA soluble (at 90°)	66 0	60 3		40 3		70 _	70 _				90 3	
Rabbit												
0 15*M* NaCl soluble				3 1		4 6	5 3	3 6		1 8		Nimni *et al* (1966)
0 45*M* NaCl soluble				3 1		9 2	6 4	8 2		1 8		
0 5*M* *citrate soluble* (pH 3 6)				3 1		7 7	7 4	_5		3 6		

Calculated from the original data (mg extractable collagen/g normal skin) using the data of Mills and Bavetta (1966) for total collagen content of rat skin (per cent of wet weight)

Collagen components in different extracts of rat skin

Mathews Kulonen and Dorfman (1954) were the first to demonstrate that denatured collagen solutions contain both monomers and large aggregates On heating its solution collagen is separated into two components Later it was shown that denatured acid soluble collagen is a mixture of two components designated α and β (Orekhovitch and Shpikiter 1955 1958) Solutions of denatured acid soluble collagen contain in addition to the α and β-components also a small proportion of a third component designated γ (Altgelt *et al* 1961 Grassmann *et al* 1961 Schlever 1962) The smallest component exist at least in certain species in three different form named $\alpha 1$, $\alpha 2$ and $\alpha 3$ with somewhat different amino acid compositions (Piez *et al* 1961 Piez 1965 Pikkarainen 1968) The $\alpha 3$-chain has not been found to exist with certainty in the collagens of higher vertebrates possibly because the $\alpha 1$ and $\alpha 3$ components are separated only with difficulty The general formula for collagen is usually expressed as $(\alpha^{2})(\alpha 1)$ During ageing intra and intermolecular cross links are introduced (see review by Harding 1965) Two intramolecularly bonded β components were identified by Piez *et al* (1961) This was followed by the isolation of a β component (Bornstein and Piez 1964) which is of intermolecular origin The γ component consists of all three types of chain linked together (Altgelt *et al* 1961 Grassmann *et al* 1961) Intermolecularly cross linked components (γ γ, β_{22} γ_{2} γ_{22} δ) have been demonstrated to exist in denatured acid soluble rat tail tendon collagen and high molecular weight bovine corium gelatin (Bornstein and Piez 1964 Veis and Anesey 1965) Alkali and salt soluble collagens contain little or none of the larger aggregates An increase of the β and γ components in the soluble collagen of rat tail tendon with age was concluded from starch gel electrophoretic patterns by Heikkinen and Kulonen (1964) and from sedimentation rates by Butzow and Eichorn (1968) A similar increase in the number of larger aggregates was observed also in rat skin collagen with age (Heikkinen and Kulonen 1964) and in consecutive extracts of rat skin (Heikkinen and Kulonen 1966) Heikkinen and Kulonen (1964) extracted rat skin at 40 and found that also a fraction of insoluble collagen containing intact components was brought into solution When skins of humans of different ages are extracted at 4 no differences are found in the proportions of α and β-components (Bakerman 1964)

The distribution of collagen components in different extract shows clearly that the existence of the β and γ-components and still larger aggregates is a characteristic of mature collagen Quantitative data on the relative proportions of different components of collagen in tissues of different ages are not available Only a small portion of the total collagen is extracted by conventional methods and a quantitative separation of collagen components of similar molecular weight is beset with difficulties A comparison of the results of different authors is handicapped by these limitations It is apparent however that the metabolic age of various collagen fractions in tissue is related to their degrees of intramolecular and intermolecular cross linking

Incorporation of labelled amino acids into different collagen fractions

Harkness *et al* (1954) were the first to examine the metabolic relationships between different collagen fraction They found that ¹⁴C glycine was most rapidly incorporated into the neutral salt soluble fraction of skin collagen of a growing rabbit The specific activity of phosphate soluble collagen rose to a maximum within 24 hours and that of

citrate-soluble collagen in 3 days. Insoluble collagen had a very low specific activity which barely changed during the experiment. Similar results were obtained by Jackson (1957) with carrageenin granulomas.

The metabolic relationship of different collagen fractions was studied also by Jackson and Bentley (1960) who found that the most highly labelled tropocollagen fraction was initially that extracted by 0.14 M sodium chloride but the peak activity was transferred within 24 hours to fractions which were extractable with 0.45 M sodium chloride and 1.0 M sodium chloride.

The ^{14}C-glycine used in the aforementioned experiment is readily incorporated into a variety of non-collagenous proteins and there is always the possibility that highly radioactive proteins contaminate collagen fractions. These objections were overcome by workers who used ^{14}C-proline or ^{14}C-lysine and subsequently isolated the corresponding hydroxylated amino acids (Gerber *et al.* 1960; Lindstedt and Prockop 1961; Prockop 1964; see review by Woessner Jr. 1968). The half-lives are shorter in the organs and tissues of young growing animals. The half-life of soluble skin collagen from a young rat was about 17 days (Kao *et al.* 1961; Gerber *et al.* 1960) and that of insoluble collagen 28—42 days. The effect of age on the turnover of collagen fractions was not studied. Three hydroxyproline pools with half-lives of 1.5 and 50—100 days have been suggested to exist in young rats (Lindstedt and Prockop 1961; Prockop 1964; Kivirikko and Prockop 1967). Urinary excretion of hydroxyproline in the older animals showed less evidence of hydroxyproline pools with short half-lives; most of the hydroxyproline excreted had a half-life of about 300 days. That the breakdown of collagen is more rapid in young than in old animals was suggested also by Laitinen (1967). The half-life of tail tendon collagen from a young rat was 20—65 days (Kao *et al.* 1961) whereas the half-life of the collagen from an adult rat was 76 days (Gerber *et al.* 1960).

These results were better understood when it was demonstrated that β- and γ-components arise from the primary subunits after their assembly into tropocollagen molecules (Martin *et al.* 1961, 1963). The effect of age on the metabolism of collagen components has not been studied. In the adult rat the specific activity of α-component is higher than that of β-components after 7 days (Martin *et al.* 1963) and even after 144 days (Orekhovitch *et al.* 1959). Larger aggregates than β-components have not been studied and these experiments have not contributed much to the clarification of the formation of insoluble collagen.

Increase of cross-links

Transformation of skin collagen with age can at present be best described in terms of intra- and intermolecular cross-links, but direct evidence of an increase in the number of cross-links is scanty. Using the method of Wiederhorn and Reardon (1952) Heikkinen *et al.* (1964) established by stress-strain measurement an increase in the number of cross-links in rat skin with age, but the nature of the cross-links remained unclear. Increased cross-linking of collagen chains with age has been reported by Heikkinen and Kulonen (1964) and Butzow and Eichhorn (1968). Soluble collagen from rat tail tendons contained increasing amounts of β- and γ-components indicating slow formation of cross-links between collagen components with advancing age.

The shrinkage of collagen fibres as a function of cross-links and age has been extensively studied by a number of workers. It has been found that the isometric tension produced during thermal contraction increases with age (see review by Verzár 1964).

Similar results have been obtained by Rasmussen *et al.* (1965) and Rigby (1964). A slight increase in the T_s of collagen with age has also been reported (Hall and Reed 1957, Brown and Consden 1958, Verzar 1964).

Changes in the physicochemical properties of collagen with age have been attributed to the formation of both covalent (see review by Verzar 1964) and noncovalent (Cross 1961) cross links with age. It is not certain yet to what extent the cross linking determines the mechanical properties of collagen fibres and which type of cross link is of primary importance in the ageing process.

Calcium and soft connective tissue

Already in 1951 Lansing *et al.* emphasized that the calcium content of the aorta wall increases with age. This was later confirmed by Verzar (1957) who proposed that the reason for the greater uptake of calcium is the change in collagen with age. The capacity to bind calcium is greater in fibres that have undergone thermal contraction. The binding of sodium, potassium and iodate to collagen fibres does not increase with age. Gustavson (1956) described the effect of different electrolytes on the shrinkage temperature of collagen and on its lyotropic properties. High concentrations (2—3 *M*) of a neutral salt, e.g. calcium chloride, promote the solubility of collagen. It has also been suggested that neutral salt competes with protein subunits for the sites of the secondary valency forces.

Microelectrophoretic studies (Gilbert 1960) have suggested that metal ions are bound to carboxyl groups of collagen. Addition of the divalent cations lead(II), copper(II), calcium(II) and magnesium(II) at constant ionic strength (0.10) increased the mobility of collagen in electrophoresis. Binding of calcium ions to citrate-soluble ox and calf tendon collagen increases above pH 3.9 (Iron and Perkins 1962), this indicates that ionized carboxyl groups are responsible for the binding.

Ichtyocol collagen contains about 68 mg of calcium and 35 mg of phosphate per gram (Clmeher 1961). When ichtyocol collagen was treated with carbobenzoxy chloride, the contents of calcium and phosphate decreased to 3.5 and 2.3 mg/g respectively and the number of free amino groups per gram of collagen decreased at the same time from 198 to 39. When ichtyocol collagen was recalcified *in vitro* after catalytic removal of the carbobenzoxy group, the amounts of calcium, phosphate and amino groups rose to the original level. The results were interpreted as showing that at least 95 % of the amino groups are not covalently bound to the metal ions and that the interaction of the majority of the amino groups and the metal ion is electrostatic. Only 50—60 % of the total lysyl and hydroxylysyl ε-amino groups of hide collagen react with dinitrofluorobenzene, whereas the proportion of such amino groups increases linearly from 60 % in untreated human dentin to more than 90 % in fully demineralized human dentin (Solomons and Irving 1958). These data suggest that a part of the amino groups in hide is involved in cross linking.

The calcium content of a purified salt-soluble collagen fraction of chick embryo was 3.14 mg/g, and that of the acetic acid-soluble collagen 10.8 mg/g (Naber *et al.* 1963). β-Aminopropionitrile, which causes the known signs of lathyrism, lowered the calcium contents of salt-soluble and acetic acid-soluble collagens to 2.0 and 2.9 mg/g

respectively Calcium copper and zinc ions partly reduced the toxic effects of the lathyrogen

Calcium ions promote the formation of insoluble collagen when rat skin slices are incubated *in vitro* (Heikkinen and Kulonen 196[illegible]) Treatment of rat tail tendon with chelating agents at pH 7 5 effects dispersion of collagen fibrils (Steven 1967), which suggests that metal ions play a part in the interaction of collagen fibrils and the interfibrillar matrix Formation of new cross links in collagen when the latter is treated with gold thiosulphate both *in vivo* and *in vitro* has been suggested by Adam *et al* (1964, 1965, 1966) Introduction of certain metal cation (Bi, Cu, Hg) into living rats caused an increase in shrinkage temperature, a prolongation of the contraction relaxation time and a decrease in the solubility of collagen (Adam and Kuhn 1968) which all indicate increased cross linking of the molecules in fibrils

Calcium ions may play a physiological part in the stabilization of collagen in soft connective tissue The mechanism of the action of calcium is still obscure The effects of other metals on collagen are probably explained by their intravital tanning properties In addition some metals apparently have an activating effect on relevant enzymes (*e g* copper on amino oxidase)

THE PURPOSE OF THE PRESENT STUDY

This study was planned to obtain knowledge on the formation of insoluble collagen in rat skin at different ages. The following approaches were chosen:

(1) Development of methods for (a) the fractionation and purification of rat skin collagen, (b) the determination of the specific radioactivity of various labelled collagen components and (c) the production of insoluble collagen in skin slices *in vitro*.

(2) Studies of the changes in the supermolecular structure, chemical composition and metabolism of collagen with age.

(3) Determination of the factors responsible for the increase in the stability of collagenous structures with special reference to the roles of metal cations and collagen-bound carbonyl groups in the formation of insoluble collagen.

MATERIAL AND METHODS

Animals and administration of labelled compounds

White rats of the Wistar strain were used. The sexes of the animals 3 to 7 days old were not determined. The animals older than 3 weeks were kept in cages of stainless steel with wide meshed bottoms. The newborn rats were kept with their dams in plastic boxes the bottoms of which were covered by wood shavings. The animals were kept on a standard laboratory diet consisting of soybean meal, maize, milk powder, margarine, sodium chloride, calcium carbonate, vegetables and pelleted rat diet (Hankkija, Finland). The content of calcium carbonate in the standard laboratory diet was about 0.3 % and that in the pelleted rat diet 1.5 %.

The labelled compounds (from The Radiochemical Centre, Amersham, England) were introduced by intraperitoneal injection. The following labelled compounds were used: TRA 82 L-Proline-T (G), CFB 17 L-Proline-^{14}C (U) and CFS 2 Calcium-45.

Incubation techniques

Preparation of skin slices. The rats were killed by a blow on a neck. The skins were removed immediately and washed in ice-cold 0.9 % sodium chloride. Hair and subcutaneous tissue were carefully removed with a razor blade. The skins were cut in a mechanical chopper (The Mickle Laboratory Engineering Co., Gomshall, Surrey, England) if not stated otherwise. In standard incubations the skin slice dimensions were 0.1 mm × 0.1 mm × the thickness of the skin.

Incubations. Most of the incubations were carried out at 37° in Erlenmeyer flasks (25—50 ml) in a metabolic shaker (A. Gallenkamp and Co. Ltd., London E.C. 2). The incubation medium which was based on the Krebs-Ringer phosphate medium, was prepared by mixing 0.20 ml of 5.75 % (w/v) KCl solution, 0.15 ml of 6.10 % (w/v) $CaCl_2$ solution

0 05 ml of 19 10 % (w/v) $MgSO_4 \cdot 7 H_2O$ solution and 5 00 ml of 0 1 M phosphate buffer pH 7 4 (Umbreit Burris and Stauffer 1957), and adding glucose to give a final concentration of 22 4 mM Ampicillin (Doctacillin® Astra Sodertalje, Sweden), 0 1 mg/ml was added to prevent bacterial growth

Duplicate samples (500 ± 25 mg each weighed either on a Mettler K7T or a Sartorius Selecta balance) of mixed skin slices were incubated in 20 ml of buffer in air if not stated otherwise Instruments used in the processing of the skin slices and glassware were sterilized at 100° for 12 hours

Isolation and purification of collagen fractions

Collagen fractions from rat skin Isolation of different collagen fractions was performed by repeated extractions with neutral salt and acetic acid solutions Collagen in the crude extracts was purified by repeated precipitation with sodium chloride and subsequent dialysis against alkaline buffers (Heikkinen and Kulonen 1966) This procedure (Fig 1) was based on modifications proposed by several authors (Jackson 1957 Gross 1958 Martin *et al* 1963 Bornstein and Piez 1964 Rubin *et al* 1965) The homogenization of the skins of old animals caused some difficulties All samples were homogenized first in a Sorvall homogenizer (Sorvall Omni Mixer OM 1961 Ivan Sorvall Inc U S A) at 16 000 rpm for 4×30 sec while the container was in an ice bath Samples were then shaken overnight at 4° and homogenized again for 2 minutes if visible particles were present In some cases it was necessary to homogenize the samples three times

Insoluble collagen from incubated skin slices The contents of the incubation flask were homogenized in a Sorvall Omni Mixer at 16 000 rpm for 2 minutes while the bottle was kept in an ice bath Samples were shaken overnight at 4° and centrifuged at 45 000×g for 30 minutes at 4° (MSE Super Speed 50S) The supernatant was discarded and the residue extracted four times overnight with 0 45 M sodium chloride at 4° The insoluble residue was dialysed against water overnight and lyophilized In some experiments as mentioned separately the extractions were continued after the extraction with 0 45 M sodium chloride either with 0 1 M citrate buffer of pH 3 6 or with 0 5 M acetic acid

Soluble collagen from incubated skin slices The contents of the incubation flask were homogenized as above The residue was extracted three times overnight with either 0 45 M sodium chloride or 0 1 M acetic acid

Fig 1 Flow sheet of the extraction and purification of different collagen fractions from rat skin If not stated otherwise the manipulation were carried out at +4 and the centrifugations in refrigerated centrifuges (MSE "High Speed" or MSE "Super Speed 50S" Measuring & Scientific Equipment Ltd, London England) at 35 000×*g* for 60 min or at 45 000×*g* for 30 min

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The skin was cut into pieces with scissors and homogenized in 0 45 *M* NaCl (5 ml/g of skin) in a rotating blade disintegrator (Sorvall Omni Mixer OM 1061 Ivan Sorvall Inc Norwalk Connecticut, U S A) for 2 min After 24 hours agitation in a swirling shaker (C Desaga GmbH Heidelberg West Germany) the mixture was centrifuged The extraction was repeated four times Homogenization was repeated between centrifugations when necessary

Residue	*Combined supernatants*
Extracted four times overnight with 0 5 *M* acetic acid and centrifuged	After filtration the collagen was precipitated with sodium chloride (final concn 15 %) overnight collected by centrifugation and dissolved in 0 1 *M* acetic acid The salt precipitation (final concn 10 %) was repeated twice Collagen was finally precipitated by dialysis first against several 0 1 *M* disodium phosphate solutions and then against water collected by centrifugation and lyophilized *Collagen soluble in 0 45 M NaCl (NSC)*

Residue of acetic acid extraction:

Residue	*Combined supernatants*
Extracted once with 0 5 *M* acetic acid at +40 for 30 min and centrifuged	Collagen purified by precipitation with sodium chloride (final concn 10 %) overnight and subsequently by dialysis first against several 0 01 *M* disodium phosphate solutions and then against water collected *by centrifugation and* lyophilized *Collagen soluble in 0.5 M acetic acid (ASC)*

Residue	*Supernatant*
Lyophilized	Lyophilized
Insoluble collagen fraction ISC_R	*Insoluble collagen fraction ISC_{40}*

Collagen in the combined supernatants was purified by precipitation with salt and dialysis against alkaline buffers as described for the neutral salt soluble fraction in Fig 1

Starch gel electrophoresis Starch gel electrophoretic separation of collagen components was conducted to get information on the components in different collagen fractions and to prepare them for the assay of their radioactivity

For the first purpose the electrophoresis was run as described by Nanto *et al* (1963 1965) under the following conditions pH 4 70, ambient

temperature +37°C ionic strength 0 017 initial voltage 120 V current 20 mA and duration of the run 5 hours

To prepare collagen components for the assay of their radioactivity it was necessary to modify the electrophoretic procedure to increase the amount of collagen separated in a single run (Fig 2)

After each electrophoresis the gel sheet was stained in a solution containing 0 1 % water soluble Nigrosine (Fluka AG Buchs Switzerland) in 50 % (v/v) aqueous glycerol on a boiling water bath for 30 minutes and then washed in 50 % aqueous glycerol The stained components were removed by cutting the spots from the gel and hydrolysed before the assay of radioactivity

In some experiments a 2 mm thick layer was cut horizontally from the surface of the gel sheet and stained The rest of the gel sheet was then cut into pieces guided by the stained pattern and the collagen components were removed from the pieces with an electric current This was accomplished by attaching the pieces of gel to the cathode end of the electrophoretic bed with 14 7 % (w/v) starch gel A dialysis bag containing about 30 ml of the standard acetate buffer was tied around the cathode end the current was connected and the collagen components were driven into the buffer solution during 3 hours The contents of the bag were centrifuged for 30 minutes at 35 000×g and the supernatant was lyophilized No electrophoretically detectable contaminants were found in the α1 and α2 components The isolation of γ components using the same technique has been described earlier (Heikkinen *et al* 1967)

Collagen content and purity of the collagen fractions Collagen

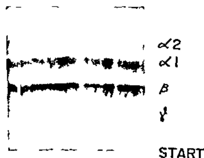


Fig 2 Separation of collagen components by starch gel electrophoresis for the assay of their radioactivity 5 mg of denatured soluble collagen prepared from the skin of a 6 day old rat was dissolved in 0 5 % acetic acid and imbibed in a 50 × 8 mm piece of Whatman 3 MM paper The filter paper was inserted in a slot at the anode end of the gel (130 mm × 62 mm × 10 mm) Electrophoresis was conducted at +3 for 5 h in a sodium acetate buffer (pH 4 60 I = 0 017) using an initial voltage of 120 V and a current of 40 mA

content was calculated by multiplying the amount of hydroxyproline present by 7.3 and protein content by multiplying the amount of nitrogen present by 5.55. The purity of the collagen fractions was estimated by comparing the collagen and protein contents. The collagen contents of the purified collagen fraction soluble in 0.45 M NaCl and the collagen fraction soluble in 0.5 M acetic acid from young rats were 92—100 %. The collagen content of the purified collagen fraction soluble in 0.5 M acetic acid from adult rats was 96—102 %.

All the collagen fractions contained varying amounts of lipids which could not be removed by the described purification process. The amount of lipids was larger in the preparations from young rats.

General analytical methods

Calcium. Calcium was determined with an atomic absorption spectrophotometer (Unicam SP 90, Unicam Instrument Ltd., Cambridge, England) using calcium chloride-lanthanum oxide standards. Samples were hydrolysed in 6 N HCl for 3 hours, the solvent was evaporated on a boiling water bath and the residue dissolved in a 1 % solution of lanthanum oxide in 25 % HCl solution. The relative standard deviation of the method was 3.4 %.

Carbonyl groups. The colorimetric assay of aldehydes was carried by a modification of the method of Paz *et al.* (1965) as follows. Lyophilized collagen samples and acetaldehyde (0.014 and 0.071 μmoles) were dissolved in 0.1 M acetic acid. N-methyl-benzothiazolone hydrazone (K & K Laboratories Inc., Calif., U.S.A.), 0.2 ml of a 1 % solution, was added to 0.8 ml of sample solution which was then kept in a boiling water bath for 3 minutes and cooled to room temperature. 2.5 ml of a 0.2 % solution of ferrous chloride was added and 6.5 ml of acetone five minutes later. The solution was mixed. The absorbance of the solution was measured at 670 nm against a reagent blank. The relative standard deviation of the method was 2.4 %.

Hydroxyproline. The hydrolyses were carried out in 6.0 N hydrochloric acid in culture tubes fitted with screw caps and Teflon packings (Kimax®) at 130°C for 3 hours. After the evaporation of the hydrochloric acid on a boiling water bath, the residue was dissolved in water. Hydroxyproline was determined by the colour reaction of Stegemann (1958) as modified by Woessner (1961). Two samples of standard gelatin (acid processed pig skin gelatin No. 149, a gift from Dr. J. E. Eastoe) were included in every series of assays. The relative standard deviation of the method is 2.2 % (Pikkarainen 1968).

Hexosamine Samples were hydrolysed in 2 *N* hydrochloric acid at 103° for 16 hours The hexosamines were freed from interfering chromogens by means of a Dowex 50 cation exchange resin according to Boas (1953) and the colour was developed by Blix's (1948) modification of the Elson Morgan method The relative standard deviation of the method was 3 2 %

Nitrogen Half a millilitre of 8 *N* sulphuric acid was added to a sample containing 5—30 μg of nitrogen and the test tube was heated at 300°C until no organic material remained 0 3 ml of water was then added, followed by 3 0 ml of Nessler's reagent (Minari and Zilversmit 1963) The absorbance was measured at 420 nm against a reagent blank after 30 minutes A series of ammonium sulphate standards was included in every digestion The relative standard deviation of the method is 2 7 % (Pikkarainen 1968)

Total protein Protein was determined by the method of Lowry *et al* (1951) as modified by Pikkarainen and Kulonen (1965) The relative standard deviation of the method is 3 1 % (Pikkarainen 1968)

Specific radioactivity of labelled hydroxyproline Two slightly different

Table III Comparison of the batch and column operations in the determination of radioactive hydroxyproline

The indicated amounts of C labelled radioactive standards were added to various inactive samples Recoveries of hydroxyproline and proline in the final eluate were measured by column operation as described by Juva and Prockop (1966) and by batch operation as explained in Table IV

Sample	Added radioactive standard		Operation	Per cent C Hyp recovered	Radioactive Pro in the final eluate (cpm)
	C Hyp dpm	C Pro dpm			
1 mg Pro + 1 mg Hyp	2580		batch	77 6	
1 mg Pro + 1 mg Hyp		13 240	batch		6
1 mg Pro + 1 mg Hyp	2580		column	81 0	
1 mg Pro + 1 mg Hyp		13 240	column		6
0 1 mg pig skin gelatin	2580		batch	79 0	
0 1 mg pig skin gelatin		13 240	batch		8
0 5 mg Hyp	2580		batch	81 2	
0 5 mg Hyp		13 240	batch		8

Mean of four determinations

Pro = proline

methods were used for the determination of the specific activity of hydroxyproline depending on the purity of the collagen fraction

The method of Prockop and Udenfriend (1960) as modified by Prockop Udenfriend and Lindstedt (1961) and further by Juva and Prockop (1966) was used to determine the specific activity of hydroxyproline in crude extracts of rat skin The direct assay method for the determination of the specific activity of hydroxyproline in purified collagen fractions was the method of Juva and Prockop (1966) modified as follows

(1) The first toluene extract was discarded This could be done without raising the content of radioactive proline in the final eluate

(2) The time consuming column operation was replaced by a simpler batch operation (Table III) The final method used mainly for the determination of the specific activity of collagen components in fractions resolved by starch gel electrophoresis is described in Table IV A similar method has been reported by Nordwig et al (1967)

Three millilitres of 'phosphor' solution (15 grams of POP and 50 mg of POPOP (Packard Instrument Company Inc Illinois U S A.) in 1000 ml of toluene) was added and the radioactivity was measured with a Tri Carb® liquid scintillation spectrometer Model 3214 (Packard In

Table IV Direct method for the determination of the specific activity of hydroxyproline in collagen components in fractions resolved by starch gel electrophoresis

1	Collagen fractions in starch gel pieces were hydrolysed in 6 N HCl at 130 for 3 h
2	Humins were removed with a humin precipitant (Prockop and Udenfriend 1960)
3	The clear filtrates were evaporated to dryness on a boiling water bath
4	The dry residue was dissolved in 8 ml of water and adjusted to pH 8 with 0 5 N NaOH and 2 ml of 0 2 M borate buffer pH 8 7 was added
5	One millilitre of 10 % alanine solution pH 8 7 was added for the stabilization of the oxidation
6	The sample was oxidized at room temperature with 2 ml of 0 2 M Chloramine T solution
7	After 20 min the reaction was stopped by adding 5 ml of 1 M sodium thiosulphate
8	The sample was kept in a boiling water bath for 30 min and then cooled to room temperature
9	Solid NaCl was added in excess
10	15 ml of toluene was added and sample was shaken for 5 min in a swirling shaker
11	After separation of the phases 14 ml of the toluene layer was transferred to a test tube
12	0 9 g of silicic acid was added to the toluene layer
13	The sample was shaken for 5 min and then centrifuged at 3000 rpm for 5 min
14	2 5 ml of the supernatant was used for Ehrlich s reaction and 8 5 ml was transferred to a counting vial for the determination of the radioactivity

strument Co) The samples were counted either up to 10 000 counts or for 100 minutes, whichever came first Samples with activities less than four times the background (25 cpm) were disregarded if not mentioned otherwise A standard sample containing 2 μmoles of hydroxyproline was present in every series of determinations The counting efficiencies were 27 % for tritium and 65 % for carbon 14 The results are expressed as dpm/μmole hydroxyproline The relative standard deviation of the method was 2 6 %

Statistical calculations

Analysis of variance and the *t* test were used to determine the significance of the difference between the turnover rates of the $\alpha 1$ and $\alpha 2$ components

A curve fitting technique was employed to compare the turnover rates of the collagen components in the skins of newborn rats The comparison was performed by applying linear regression analysis which was carried out on an IBM 1130 computer in the Department of Applied Mathematics, University of Turku by Mr Heikki Copponen M Sc

RESULTS

Supermolecular structures of rat skin collagen at different ages

Collagen fractions

The collagen content of rat skin (100×weight of collagen/fresh skin weight) increased with age as follows 1 73 % in 6 day old rats 2 24% in 2 week old rats 13 90 % in 8 week old rats and 20 10 % in 6 month old rats The proportions of 0 45 M sodium chloride soluble 0 5 M acetic acid soluble and insoluble collagens in rat skin varied with age as shown in Fig 3 The skin of a newborn rat is characterized by a high content of insoluble collagen During the rapid growth period mainly acetic acid soluble collagen accumulates

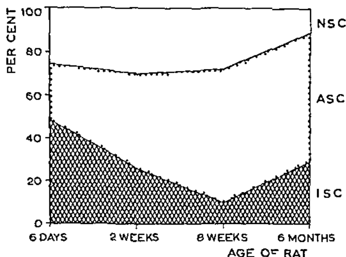


Fig 3 Effect of age on the relative proportions of various fractions of rat skin collagen Neutral salt soluble (NSC) and acetic acid soluble collagen (ASC) were isolated by repeated extraction with 0 45 M NaCl and 0 5 M acetic acid respectively using the t l l procedure (Fig 1) Pooled skins from at least 3 animals of the indicated age were fractionated

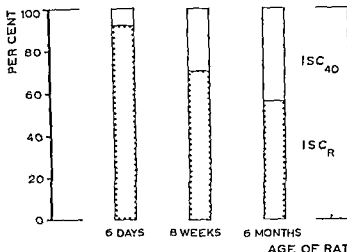
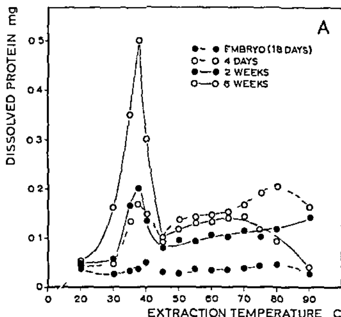


Fig 4 Proportions of ISC₄₀ and ISC in the skins of rats of different ages For the preparation of th fractions of insolubl collagen s e Fig 1 Pooled kins of at l ast 3 animals were u e l in the fractionations

Insoluble collagen yields on heating in 0 5 M acetic acid at 40° for 30 minutes a soluble fraction designated ISC_{40} The amount of this fraction increases with age (Fig 4) When extracted stepwise at temperatures in the range 20°—90° insoluble collagen of the adult rat dissolves at lower temperatures than that of the young rat (Fig 5) This difference is especially apparent when comparisons are made between 5 day old and 24 year old rats About 50 % of the insoluble collagen from the adult rat is dissolved at 40° More than half of the insoluble residue of the skin of a 5 day old rat remains undissolved even after gelatinization at 120° for one hour The insoluble collagenous residue from a 24 year-old rat is dissolved almost completely by such treatment The biphasic solubility of the insoluble collagen with rising temperature is apparent

When rat skin is extracted consecutively with solvents of increasing ionic strength and diminishing pH the proportion of larger aggregates of collagen components increases (Fig 6) ISC_{40} from the skin of an old rat contains more β and γ-components than the corresponding fraction from the skin of a young rat (Fig 7)



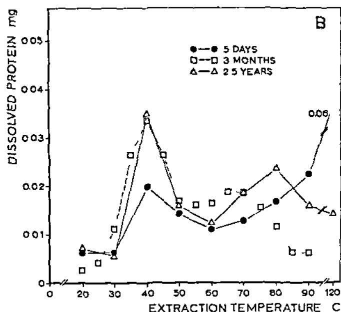


Fig 5 Heterogeneity of insoluble collagen of rat skins of different ages Soluble collagens were removed from rat skins of the indicated ages by extraction with 0 45 *M* NaCl and 0 5 *M* acetic acid (Fig 1) The residues were then extracted consecutively with 0 5 *M* acetic acid at the indicated temperatures for 15 min and centrifuged between the extractions at 35 000×*g* for 30 min in experiment A and at 45 000×*g* for 30 min in experiment B

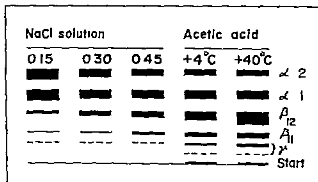


Fig 6 Collagen components in consecutive extracts of rat skin The indicated collagen fractions were obtained from skins of rats (20—30 g) using the standard procedure (Fig 1) Lyophilized fractions were dissolved in 0 1 *M* acetic acid and resolved into components by starch gel electrophoresis

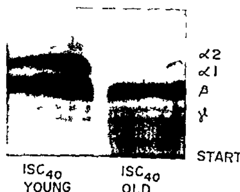


Fig 7 Starch gel electrophoretic patterns of ISC_{40} fractions of young and old rats Soluble collagens were removed by repeated extraction first with 0 45 *M* NaCl and then with 0 5 *M* acetic acid (Fig 1) The residue was then extracted with 0 5 *M* acetic acid at 40 to obtain ISC_{40} Young = 6 day old rats old = 2½ year old rats

Chemical compositions of the collagen fractions

The nitrogen hydroxyproline and hexosamine contents of different collagen fractions from rat skin vary with age (Table V) The ratio of hexosamine to hydroxyproline in ISC_R decreases rapidly with age ISC_{40} from the skin of an adult rat is almost pure collagen according to the amino acid composition (glycine 319 proline 135 hydroxyproline 82 residues/ 1000 residues and traces of tyrosine) *

The author is grateful to Dr J Iikkarainen for the determination of the amino acid composition

Table V Hydroxyproline nitrogen and hexosamine in various fractions of rat skins of different ages

Skin fractions were isolated by repeated extraction with 0 15 M sodium chloride and with 0 5 M acetic acid until the protein content of the supernatant was less than 0 02 mg/ml The residue was extracted further with 0 5 M acetic acid for 30 min at 40 to obtain ISC_{40}

Skin fraction	Collagen / Total protein × 100	The ratio of hexosamine to hydroxyproline (w/w)
0 15 M NaCl soluble fraction		
from 6 day old rats	4 9	0 63
from 8 week old rats	40 5	0 05
from 6 month old rats	43 2	0 07
0 5 M acetic acid soluble fraction		
from 6 day old rats	30 1	0 10
from 8 week old rats	99 2	*0 01*
from 6 month old rat	99 8	0 01
ISC_{40}		
from 6 day old rats	40 0	—
from 8 week old rats	93 3	—
from 6 month old rats	111 2	—
ISC_R		
from 6 day old rats	16 4	0 47
from 8 week old rats	98 9	0 02
from 6 month old rats	102 5	0 01

It can be concluded from the data that the chemical composition of the insoluble fraction of rat skin collagen varies with age ISC from a young rat contains large amounts of non collagenous proteins and carbohydrates The ISC_{40} which contains large amounts of β and γ components increases with age which suggests that interactions between collagen components are largely responsible for the decreased solubility of collagen in the skin of an adult rat

If aldehydes take part in the cross linking of collagen, there should be smaller numbers of free aldehyde groups in the insoluble forms of collagen To test this hypothesis the aldehyde group contents of subfractions of the ISC of 2½ year old rats were determined The content of aldehyde groups was lower in the fractions extracted at 70°—80° (2 44 acetaldehyde equivalents/TC) than in the fractions extracted at 20°—60° (3 60 acetaldehyde equivalents/TC) A loss of one aldehyde group per mole of tropocollagen is apparent (Table VI)

Table VI Aldehyde group contents of subfractions of insoluble collagen

Insoluble collagen was isolated from skins of 2½ year old rats It was then extracted with 0 5 *M* acetic acid for 15 min consecutively at the indicated temperatures

Temperature	Dissolved protein mg	Per cent collagen	Aldehydes TC
20	11 45	105 1	3 79
30	8 42	103 2	3 60
40	73 10	102 2	3 48
50	24 10	95 8	3 68
60	14 10	101 5	3 43
70	17 10	95 6	2 50
80	31 40	109 5	2 51
90	25 05	106 5	2 30

Free aldehyde groups in the soluble collagens of normal and lathyritic rats were also determined to find out the effect of administration of aminoacetonitrile and semicarbazide on collagen Aminoacetonitrile and semicarbazide when given to growing rats (initial weights 90—100 g) in the diet during two weeks caused the known signs of lathyrism (cessation of growth increase in the amount of soluble collagen) Different collagen

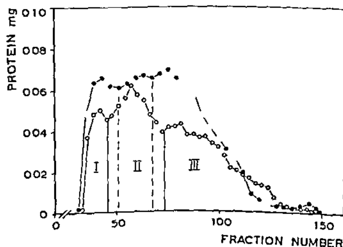


Fig 8 Fractionation of normal and lathyritic collagens by gel filtration 100 mg of 0 5 *M* acetic acid soluble collagen was denatured at 40° for 15 min and fractionated on a Sephadex G 200 column at room temperature using 0 5 % acetic acid as eluent ●—● acetic acid soluble collagen from lathyritic (semicarbazide) rats, O—O acetic acid soluble collagen from normal rats The experimental conditions are described in the legend of Table VII

fractions were isolated and purified by the standard procedure (Fig 1). Collagens soluble in 0.5 *M* acetic acid were further fractionated on Sephadex G 200 columns (Fig 8).

The aldehyde group contents of the 0.45 *M* NaCl soluble and 0.5 *M* acetic acid soluble collagens are tabulated in Table VII. The aldehyde

Table VII Aldehyde group contents of collagen fractions from normal and lathyritic rats

Aminoacetonitrile (75 mg/100 g/day) or semicarbazide (30 mg/100 g/day) was included in the diet for [illegible] weeks. 0.45 *M* NaCl soluble and 0.5 *M* acetic acid soluble collagens were isolated and purified (Fig 1) and the acetic acid soluble collagens were further fractionated by gel filtration as described in the legend of Fig 19.

Fraction	Aldehydes/TC			Inhibition by semicarbazide %
	Aminoaceto nitrile	Semi carbazide	Control	
NSC	0.87	0.93	1.47	37
ASC	2.01	2.31	3.39	3[illegible]
Sephadex G 200				
Fraction I	—	2.55	2.70	6
Fraction II	—	1.86	3.39	45
Fraction III	—	1.68	4.26	61

Table VIII Effect of lathyrism on the calcium content of rat skin collagen

The collagen fractions are the same as described in Table VII. One sample of each fraction was hydrolysed in 6 N HCl for 3 h at 130°, evaporated to dryness and dissolved in a 1 % solution of lanthanum oxide in 25 % HCl solution.

Fraction	Moles of calcium / Mole of tropocollagen	Decrease %
NSC		
Aminoacetonitrile	24.9	31
Semicarbazide	2[illegible].0	[illegible]5
Control	36.0	0
ASC		
Aminoacetonitrile	21.9	—
Semicarbazide	20.5	—
Control	17.1	—
ISC		
Aminoacetonitrile	14.4	21
Semicarbazide	17.1	6
Control	18.2	0

group contents of lathyritic collagens are about half of those of normal collagens In the fractions I, II and III resolved on Sephadex G 200 which roughly correspond to the γ β and α components of collagen the aldehyde group content was highest in the normal α component (Table VII) In lathyritic collagen the opposite was true this suggests that the aldehyde groups participate in the formation of interchain cross links

Because calcium ions promoted the formation of insoluble collagen *in vitro* (Fig 15) the amounts of calcium ions bound to collagens isolated from normal and lathyritic rats were determined (Table VIII) The calcium content varied from 14 4 to 36 0 moles per mole of tropocollagen depending on the collagen fraction The fact that the 0 45 M NaCl soluble collagen from lathyritic rats contained less calcium than the corresponding fraction from normal rats suggests that lathyrogens may affect the metabolism of collagen in several ways

Incorporation of labelled proline into the collagen fractions

The incorporation of labelled proline into collagen varies with age (Fig 9 and Table IX) Labelling of the acetic acid soluble and insoluble collagens

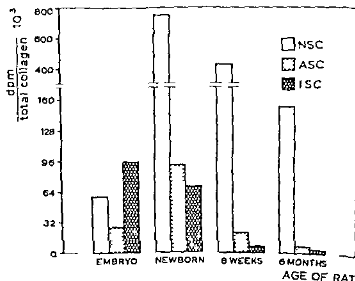


Fig 9 Effect of age on the incorporation of labelled prolin into collagen of rat skin H Proline (0 5 μCi/g into pregnant rats and 1 μCi/g into the others) was injected intraperitoneally into rats of the indicated ages After 4 h the rats were killed and different collagen fractions of the skins were isolated by the standard procedure (Fig 1)

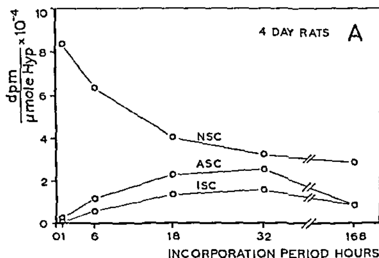
Table IX The incorporation of labelled proline into collagen of rat skin at different ages

The experimental conditions were those described in the legend of Fig 9 The values are the means of three determinations performed on pooled skins The ratios were calculated from the specific radioactivities of hydroxyproline

Animals	No of animals	^{3}H Hydroxyproline, dpm per g of fresh skin	ASC/NSC	ISC/NSC	ISC/ASC
6 day old rats	8	140 000	0 040	0 085	1 213
8 week old rats	4	91 500	0 015	0 013	1 114
6 month old rats	3	,1 000	0 004	0 00_	0 79_

takes place faster in the skin of a young than in the skin of an adult rat This suggests that not only the synthesis of collagen but also the conversion of soluble collagens into insoluble form occur at a higher rate in young than in adult rats

The half lives of 0 45 M NaCl soluble collagens of 6 day old and 3 month old rats were 27 and 73 hours respectively (Fig 10) The metabolic turnover rate of the collagen components in the 0 1 M acetic acid soluble fraction of the skins of 6 day old rats shows that the $\alpha 2$ component has a higher radioactivity during the first 24 hours after the injection of labelled



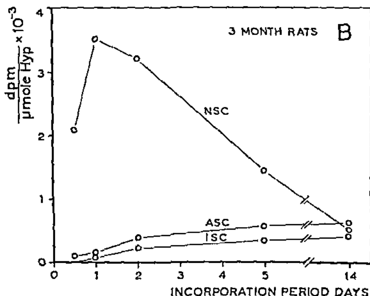
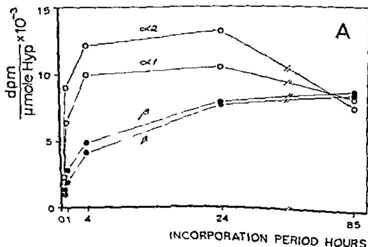


Fig 10 Turnover of different collagen fractions in skins of young and adult rats H Proline was injected intraperitoneally into 4 day old (2 μCi/g A) and 3 month old (1μCi/g B) rats After the indicated incorporation periods the rats were killed and the collagen fractions were isolated (Fig 1)

proline (Fig 11) The specific activities of the β and γ components are high already after an incorporation period of 0 5 hour this suggests that these components are of intracellular origin After an incorporation period of one hour the specific activity of the β and γ components rises less rapidly than that of the α components This probably indicates that the β and γ components are formed from α chains also in the extracellular space



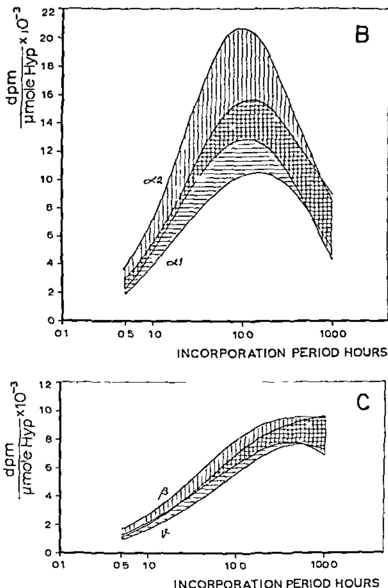


Fig 11 Turnover of collagen components in the skins of 6 day old rats ^{3}H Proline (1 µCi/g) was injected intraperitoneally into 6 day old rats After the indicated incorporation periods the rats were killed Collagen soluble in 0 1 M acetic acid was isolated by four extractions purified and resolved electrophoretically into components as described in the experimental section

Each datum in Fig 11 A is the mean of four determinations Analysis of variance and t tests on the values of 1 4 and 24 h revealed a statistically significant difference between the turnover rates of the α components ($F(1,18) = 2$[illegible]3 $P < 0.001$ $t(11) = 8$[illegible]5 $P < 0.001$)

A curve fitting technique was employed to compare the turnover rates of the α components (B) and the β and γ components (C) The shaded areas represent 95 % confidence regions

The fact that the specific activity of the α components is a maximum after 10 hours points to a high metabolic rate of collagen components in the skins of 6 day old rats

Formation of insoluble collagen *in vitro*

Redistribution of radioactive hydroxyproline in different collagen fractions during incubation

Soluble collagens became insoluble when skin slices were incubated in Krebs Ringer phosphate medium *in vitro* This was seen as an increase in the amount of radioactive hydroxyproline in the insoluble collagen in the skins of 6 day old rats during incubation (Fig 12 Table X) The initial fall in the radioactivity of the 0 45 *M* NaCl soluble collagen is mainly due to the dissolution of collagen in the incubation medium (Fig 14) The specific radioactivity of the insoluble fraction of incubated skin collagen remained unchanged after extraction with a citrate buffer (pH 3 6 0 1 *M*) or after gelatinization at 120° for 3 hours and subsequent dialysis against running tap water *This indicates that soluble collagen macromolecules*

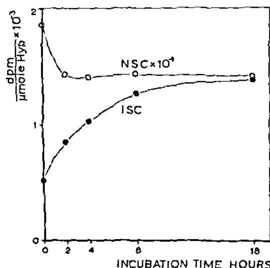


Fig 12 Formation of insoluble collagen in skin slices in vitro ³H Proline (1μCi/g) was injected intraperitoneally into 6 day old rats After 4 h the rats were killed the skins were cut into slices and incubated in glucose containing Krebs Ringer phosphate medium at 37° for the indicated times (for details, see p 21)

Table X Formation of insoluble collagen in skin slices in vitro

^{3}H Proline (1 μCi/g) was injected intraperitoneally into 7 day old rats After 4 h, the rats were killed and skin slices 0.5 mm thick were incubated in glucose-containing Krebs Ringer phosphate medium for the indicated period The specific radioactivity of hydroxyproline is expressed as dpm/mg of collagen

Sample	Incubation period h			
	2	4	6	8
NSC	25 000	18 550	17 400	17 050
ASC	1 350	1 590	2 200	2 830
ISC_{40}	1 430	1 770	2 200	2 800
ISC_R	1 340	1 265	1 610	1 750

were converted into insoluble form during the incubation and not only adsorbed

The formation of insoluble collagen was accelerated by a rise in the incubation temperature (Fig 13) When skin slices were incubated at different pH, it was found that the proportion of insoluble collagen was lowest near pH 6 4 and increased both above and below this pH (Fig 14) The formation of insoluble collagen was more rapid in thin slices

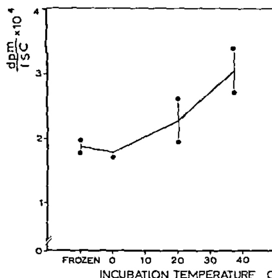


Fig 13 Effect of incubation temperature on the formation of insoluble collagen in skin slices in vitro The incorporation period was 4 h Otherwise the experimental conditions were the same as described in the legend of Fig 12 The bars represent the ranges of three values

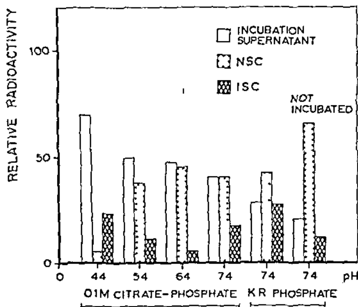


Fig 14 Effect of pH on the formation of insoluble collagen in skin slices in vitro Labelled skin slices (see Fig 1.) were incubated either in a 0 1 *M* citrate phosphate buffer or in a glucose-containing Krebs Ringer phosphate medium for 6 h The contents of the incubation flasks were then centrifuged at 45 000×*g* for 60 min Neutral salt soluble collagen was extracted from the sediment with 0 45 *M* NaCl (Fig 1) The incubation supernatants 0 45 *M* NaCl soluble fractions and the insoluble residue were dialysed overnight against running water and lyophilized

Table XI Effect of the thickness of skin slices on th formation of insoluble collagen in vitro

Labelled slices of different thickness were cut with a mechanical chopper and incubated under the standard conditions for 5 h All the incubations were performed in duplicate

Thickness mm	³H Hydroxyproline fpm/ISC	
	Exp 1	Exp 2
0 1	106 000	14 000
0 3	—	15 700
0 5	8. ,00	—
1 0	70 100	9 200
1 0 (not incubated)	56 500	—

(Table XI) This indicates that one or more of the components of the incubation medium necessary for the formation of the insoluble collagen When skin slices were incubated *in vitro* after different periods of in

Table XII Effect of the length of the incorporation period in vivo *on the increase in radioactivity of ISC during the subsequent incubation* in vitro

³H Proline (1 μCi/g) was injected intraperitoneally into 6 day old rats After the indicated incorporation periods the rats were killed and skin slices were incubated for 5 h All the incubations were carried out in duplicate

Incorporation period *in vivo* h	Incubation *in vitro*	³H Hydroxyproline dpm/μmole	Increase %
1	—	920	138
	+	2190	
4	—	1720	165
	+	4560	
8	—	2750	6
	+	4460	
27	—	7200	32
	+	9480	

corporation of labelled proline *in vivo* the per cent increase in the radioactivity of insoluble collagen was a maximum after an incorporation period of 4 hours (Table XII)

It is obvious that insoluble collagen is formed during the incubation of skin slices *in vitro* This provides a method for the investigation of the mechanism of formation of insoluble collagen *in vitro* which is described in the following section

Effect of metal cations on the formation of insoluble collagen in vitro

Calcium ions in the incubation medium promoted the formation of insoluble collagen (Fig 15) whereas EDTA in 5—10 mM concentrations inhibited the formation of insoluble collagen almost completely (Fig 16) The effect of calcium ions was observed in the pH range 4 4—8 4 Formation of insoluble collagen occurred also in citrate phosphate buffers on both sides of pH 6 4 without metal cations present (Fig 14) These observations suggest that the formation of insoluble collagen *in vitro* depends on the number of charged groups in the tropocollagen macromolecule

To study the possible binding of calcium ions to collagen during incubation calcium 45 was injected into 6 day old rats After an incorporation

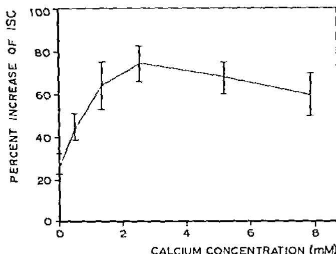


Fig 15 Effect of calcium ions on the formation of insoluble collagen in skin slices in vitro Labelled skin slices (Fig 1°) were incubated for 6 h in Krebs Ringer phosphate medium containing glucose and varying concentrations of cal ium The bars represent the ranges of three values

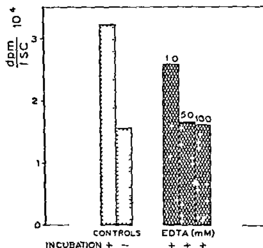


Fig 16 Effect of EDTA on the formation of insoluble collagen in skin slices in vitro Labelled slices (see Fig 12) were incubated for 6 h in Krebs Ringer phosphate medium containing gluc e and different concentrations of EDTA Duplicate incubations were performed at each concentration

Table XIII Effect of incubation on the incorporation of calcium 45 into insoluble collagen

Calcium 45 (10 μCi/rat) was injected intraperitoneally into 6 day old rats and after incorporation periods of 4 and 27 h the rats were killed and skin slices were incubated under the standard conditions for 6 h All the incubations were performed in duplicate

Incorporation period in vivo	Incubation in vitro	Calcium 45 dpm/ISC	
		Mean	Range
4 h	—	4 670	4 405— 4 935
	+	10 640	8 990—1 300
24 h	—	7 220	6 345— 8 095
	+	24 700	17 600—31 800

Table XIV Effect of metal cations on the formation of insoluble collagen in skin slices in vitro

³H Proline was injected intraperitoneally into 6 day old rats After 4 h the rats were killed and skin slices were incubated in Tris HCl buffers containing metal cations (0 15 M pH 4) at 37 for 5 h Metal cations were added as chlorides (lead as acetate) to a concentration of 2 6 mM

Experiment	Amount of injected ³H proline	Cation	³H Hydroxyproline dpm/μmole	
			Mean	Range
No 1	3 μCi/g	Li	2240	2 30— 50
		Au	31 5	2960—3 90
		Ca	2905	2820—3180
		Sr²	2395	2340—2430
		Ba²	2385	2380— 3 0
		Hg	3110	2 90—3430
		Fe	3055	3260— 0
		Co	3 60	3300—3 90
		Ca	19 0	1860— 030
No 2	1 μCi/g	Cd²	1 95	1200—1390
		Bi	1150	1130—1170
		Cr³⁺	1 90	1265—1310
		Pb	10 0	1040—1100
		Sn⁴	1050	1020—1090
		Ca	845	—
No 3	2 μCi/g	Cu	2670	2610— 30
		Cu²	2 85	2740— 8 0
		Ca	2300	2210—2390
		Al	2 05	2090—2 90
		Fe²	2280	2180— 380
		Fe	425	23 0— 500
		Ni	2 00	2 30— 0
		Ca	1 40	1420—1660

period of 4 hours, skin slices were incubated at 37° C for 5 hours The radioactivity of the insoluble collagen increased during the incubation (Table XIII)

A number of different metals were added as their chlorides to the incubation medium (Table XIV) Cobalt(II), copper(II) copper(I) and gold(III) ions had the most marked effect The incubations with the cations present were carried out mainly in a 0 15 M Tris HCl buffer of pH 7 4 because some of the metals (*e g* Al^{3+}, Ba^{+}, Co^{+} Cu , Fe^{3+}) were precipitated by the phosphate in the Krebs Ringer phosphate medium The effects of the metal cations were greater in the Krebs Ringer phosphate medium The effects of different metals are seen in Table XV

Table XV Comparison of the effects of various cations on the formation of insoluble collagen in vitro

Average relative increases in the radioactivity of hydroxyproline of insoluble collagen during incubation calculated from the results presented in Table XIV

Cation	Increase	Cation	Increase
Co	82 5	Fe^{3}	48 0
Cu	80 7	Al	43 1
Cu	3 4	Bi	36 0
Au	6[illegible] 8	Pb	26 6
Ni	6[illegible] 3	Sn^{4+}	24 3
Hg	59 4	Sr^{2}	22 3
Fe	[illegible]6 7	Ba	[illegible]2 3
Cr	5[illegible] 7	Mn^{2}	20 2
Ca^{2+}	[illegible]0 4	Mg^{2}	20 1

The formation of insoluble collagen *in vitro* was not dependent on the availability of molecular oxygen (Table XVI) Anaerobic conditions, sodium fluoroacetate or sodium fluoride or the absence of glucose did not inhibit the incorporation of hydroxyproline into insoluble collagen during the incubation

Attempts to influence the formation of insoluble collagen with *αα* dipyridyl, thioglycolic acid pyridoxine and 4-desoxypyridoxine (all in concentrations of 0 1 and 1 0 mM) were not successful Also an inhibition of protein synthesis by puromycin during the *in vitro* phase had no effect on the radioactivity of insoluble collagen Freezing and thawing of the skin slices did not inhibit the formation of insoluble collagen during subsequent incubation *in vitro*

Table XVI Formation of insoluble collagen in skin slices of rats of varying ages when the skin slices were incubated under different atmospheres

^{3}H Proline was injected intraperitoneally into rats of varying ages (1 μCi/g, 3 rats per group) After an incorporation period of 4 h the rats were killed and the sliced skin samples were incubated for 6 h in glucose containing Krebs Ringer phosphate medium under the indicated atmospheres

Age of rats	Atmosphere	Incubation	^{3}H Hydroxyproline, dpm/μmole			Increase %
			NSC	ASC	ISC	
6 days	Air	—	25 700	1510	2100	
	95 % O—5 % CO	+	17 900	[illegible]580	3690	76
	N$_2$	+	17 100	[illegible]390	3470	65
6 weeks	Air	—	3 390	64	352	
(male)	95 % O—5 % CO	+	2 460	144	440	25
	N$_2$	+	2 350	152	482	39
6 months	Air	—	4 610	16	32	
(male)	95 % O_2—5 % CO_2	+	2 150	24	42*	(31)
	N$_2$	+	1 900	24	48	(50)

* Radioactivities less than 100 cpm (background 25 cpm)

Formation of insoluble collagen in skin homogenates

Formation of ISC occurred also in skin homogenates, but was less rapid if the homogenate was first centrifuged at 40 000×g for 30 minutes the

Table XVII Formation of insoluble collagen in rat skin homogenates during incubation in vitro

H Proline was injected intraperitoneally into 6 day old (Exp 2) and 3 day old (Exp 1) rats After an incorporation period of 4 h the rats were killed and their skins were cut into slices One part of the pooled slices was homogenized and an aliquot of the homogenate centrifuged for 30 min at 45 000×g Skin slices the total homogenate and the 45 000×g sediment were incubated in 0 15 M Tris HCl buffer pH 7 4, for 5 h

Experiment	Sample	H Hydroxyproline dpm/μmole		Increase %
		Mean	Range	
No 1	Slices	25 700	24 400—27 000	49
	Homogenate	25 900	26 800—27 000	51
	Unincubated slices	17 200	16 800—17 600	0
No 2	Slices	6 000	5 440— 6 560	100
	Homogenate	6 240	[illegible]10— 6 7[illegible]0	108
	45 000×g sediment	5 480	4 880— 6 080	83
	Unincubated slices	3 000	2 400— 3 600	0

supernatant removed and the sediment incubated *in vitro* under the standard conditions The results of the experiments with skin homogenates are summarized in Table XVII The formation of insoluble collagen was similar in homogenates prepared from whole embryos and from skins of 6 day old rats Addition of embryonic supernatant ($35\,000 \times g$) to the incubation medium did not affect the formation of insoluble collagen in skin slices (Table XVIII)

It can be concluded that insoluble collagen is formed also in skin homogenates *in vitro* and that this collagen is mainly found in the $45\,000 \times g$ sediment

Table XVIII Effect of embryonic supernatant on the formation of insoluble collagen in skin slices in vitro

Rat embryos (16—17 days) were homogenized in the Krebs Ringer phosphate medium in a Sorvall homogenizer for 2 min and the homogenate was centrifuged for 30 min at $35\,000 \times g$ *^{3}H Proline (1 µCi/g) was injected intraperitoneally into 6 day old rats of the* indicated ages In experiments nos 1 and 2 the rats were killed after 5 h in experiment no 3 after 3 h The skins were cut into slices and incubated under the indicated conditions After 4 h the incubations were interrupted the media were decanted fresh media were added and the incubations continued for a further 4 h before the radioactivity was measured

Experiment	Addition to the medium	Incubation	Hydroxyproline dpm/µmole	Increase %
No 1 (6 day old rats)	—	—	2750	—
	—	+	4110	50
	Embryonic supernatant ($35\,000 \times g$)	—	2960	8
	Embryonic supernatant ($35\,000 \times g$)	+	3800	38
No 2 (4 week old female rats)	—	—	208	—
	—	+	510	145
	Embryonic supernatant ($35\,000 \times g$)	+	376	81
	Boiled embryonic supernatant ($35\,000 \times g$)	+	405	95
	Boiled embryonic supernatant ($35\,000 \times g$)	—	256	20
No 3 (8 week old male rats)	—	—	320	—
	—	+	632	97
	Embryonic supernatant ($35\,000 \times g$)	+	800	150

Role of aldehyde groups in the formation of insoluble collagen in vitro

When semicarbazide or phenylhydrazine was added to the incubation medium, the formation of insoluble collagen was retarded (Fig 17) Semicarbazide and aminoacetonitrile also inhibited to some extent the synthesis of collagen when skin slices were incubated in Krebs Ringer phosphate medium of pH 7 4 in the presence of ^{3}H proline The formation of α , β and γ components was inhibited to the same extent (Table XIX)

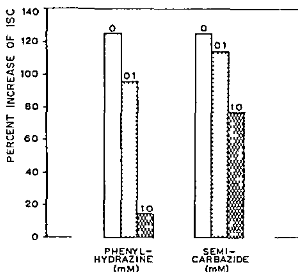


Fig 17 Effect of semicarbazide and phenylhydrazine on the formation of insoluble collagen in skin slices in vitro Labelled slices (Fig 12) were incubated for 6 h in the Krebs Ringer phosphate medium containing varying concentrations of semicarbazide or phenylhydrazine Duplicate incubations were performed in each treatment

To study the metabolism of carbonyl groups skin slices from a 6 day old rat were incubated under different conditions and the carbonyl groups in the 0 45 *M* NaCl soluble fraction were determined as described in Table XX Carbonyl groups were not formed in the absence of glucose, but their synthesis was not inhibited under anaerobic conditions

These data suggest that carbonyl groups are involved in the formation of insoluble collagen *in vitro* The mechanism of their action is not known

Table XIX Effect of aminoacetonitrile and semicarbazide on the synthesis of collagen components in vitro

Skin slices from 6 day old (Exp 1) and 4 day old (Exp 2) rats were incubated in glucose containing Krebs Ringer phosphate for 4 h 100 μCi of ^{3}H proline was added to 20 ml of the medium containing 700 mg of skin slices in Exp 1 and 500 mg in Exp 2 The concentrations of aminoacetonitrile and semicarbazide were 5 0 mM Duplicate incubations were performed in each treatment After the incubation the mixtures were homogenized in the incubation medium for 2 min in the Sorvall homogenizer and centrifuged for 30 min at 45 000×g The residues were extracted with 0 1 M acetic acid overnight and centrifuged as above The combined supernatants were dialysed against water lyophilized and the collagen was dissolved in 0 1 M acetic acid 2 5 mg of rat tail tendon collagen was added as an internal standard to 1 ml of the collagen solution the collagen components were separated by starch gel electrophoresis and the specific radioactivities of hydroxyproline were measured

Experiment	Collagen fraction	^{3}H Hydroxyproline dpm/μmole		Inhibition %
No 1		Aminoacetonitrile	Control	
	α components	530	1290	59
	β-components	390	675	42
	γ components	880	1860	47
	ISC	760	3200	76
No 2		Semicarbazide	Control	
	α-components	5200	8000	35
	β components	1140	1910	40
	γ components	1860	3000	38

Table XX Increase in aldehyde group content of the 0 45 M NaCl soluble fraction of rat skin during incubation

Skin slices of 6 day old rats were incubated for 4 h under the indicated conditions After incubation the neutral salt soluble fraction was isolated by three extractions with 0 45 M sodium chloride dialysed against water lyophilized and dissolved in 0 1 M acetic acid

Medium	No of replicate	Atmosphere	Incubation	μmoles of aldehyde / 300 mg of protein	
				Mean	Range
Krebs Ringer phosphate	5	Air	—	2 08	1 77—2 34
Krebs Ringer phosphate	4	Air	+	2 19	1 95—2 [illegible]1
Krebs Ringer phosphate + glucose	5	Air	+	2 90	2 70—3 15
Krebs Ringer phosphate + glucose	2	[illegible]	+	2 91	2 77—2 85

4

DISCUSSION

The soluble and insoluble collagens of rat skin

Changes in the solubility of skin collagen with age

Soluble collagens The changes in the amount of 0 45 *M* NaCl soluble collagen with age (Fig 3) are in agreement with earlier results (Mills and Bavetta 1966 Tsurufuji and Nakagawa 1967) Only small quantities of collagen are dissolved by acid buffer solutions after the extractions with sodium chloride solution (Mills and Bavetta 1966) Different results are obtained when 0 5 *M* acetic acid is used for extraction Up to 80 % of skin collagen can be brought into solution when skin is extracted first with 0 45 *M* sodium chloride and then with 0 5 *M* acetic acid (Heikkinen and Kulonen 1966 Fig 3)

These results show that the solubility of collagen is a matter of definition Such terms as *procollagen* (Orekhovitch 1953) *alkali soluble collagen* (Harkness *et al* 1954) *neutral salt soluble collagen* (Gross *et al* 1955 Jackson and Fessler 1955) and *acid soluble collagen* do not define satisfactorily a given collagen fraction More exact operational definitions *e g* 0 45 *M* NaCl soluble collagen instead of neutral salt soluble collagen and 0 5 *M* acetic acid soluble collagen instead of acid soluble collagen are necessary Gel electrophoretic patterns and their densitometric tracings give more specific information about the molecular structures of collagens in solution as indicated by Nanto *et al* (1963) The collagen insoluble in 0 45 *M* sodium chloride but soluble in 0 5 *M* acetic acid accumulates in rat skin during the growth of the animal (Fig 3) and contains more β and γ components than collagen soluble in 0 15—0 45 *M* sodium chloride (Fig 6) The bonds that keep the molecules in lateral alignment are disrupted by acetic acid This indicates that most of the collagen molecules in adult rat skin are united by labile intermolecular bonds

Insoluble collagen In the present study insoluble collagen was defined as the fraction of skin which did not dissolve in 0 45 *M* sodium chloride and

0 5 M acetic acid at 4° The amount of this fraction was largest in the skin of a newborn rat (Fig 3) Fractionation of the insoluble collagen suspended in 0 5 M acetic acid by raising the temperature stepwise gave an unexpected biphasic solubility curve (Fig 5) The fraction of insoluble collagen that dissolved in 0 5 M acetic acid at 40° was designated ISC_{40} and the residue ISC_R

It is obvious that the only satisfactory way to describe insoluble collagen is operational as in the case of soluble collagens *Collastromin* (Tustanovski *et al* 1954), *metacollagen* (Banga *et al* 1956) and *eucollagen* (Courts 1960) which have been proposed for insoluble collagen have not gained general acceptance

ISC_{40} It was shown by Pikkarainen and Kulonen (1964) that intact α and β components can be released from insoluble collagen by mild thermal denaturation The amount of large aggregates (β γ, δ) in rat skin collagen that dissolves in acid buffers at 40° increases with age (Heikkinen and Kulonen 1964) In the present study it was observed (Fig 4) that the amount of the ISC_{40} fraction in rat skin increases with age According to its amino acid composition this fraction is almost pure collagen in an adult rat (p 31)

It appears that one form of insoluble collagen in rat skin is characterized by a large number of cross links between α chains About 70 % of the collagen in the skin of an adult rat consists of various bonded components (Figs 3 4 6 and 7) In addition to intramolecularly bound components the ISC_{40} fraction from adult rat skin contains also intermolecularly bonded polymers (Fig 7) In bovine corium matrix gelatin, the intermolecularly bonded components comprise about 60 % and the intramolecularly bonded components less than 20 % of the total aggregates (Veis 1967) Intramolecularly bonded components amounted to 42 % of the acid extracted heat denatured rat tail tendon collagen and at most 27 % of the components were intermolecularly bonded

The nature of the forces which bind the collagen molecules to supermolecularly organized aggregates in the ISC_{40} fraction is not clear Two kinds of swelling occur in an acid collagen solution depending on the temperature (Rigby 1967) Below a characteristic temperature which for rat collagen is about 36° the swelling is reversible but above the melting point of tropocollagen (36°) the swelling is irreversible The possibility exists that some of the intermolecular bonds are connected to collagen bound aldehyde groups This will be discussed in more detail on page 56

ISC_R The point to consider next is the insoluble collagen matrix which does not dissolve in 0 5 M acetic acid at 40° This fraction is large in the

skin of a young rat (Fig. 4). The collagen in the ISC_R fraction from a young rat amounts to only about 16 % of the total skin proteins. The corresponding fraction of adult rat skin contains only small amounts of non-collagenous proteins (Table V). The hexosamine content of the ISC_R fraction decreases with age (Table V). The ISC_{40} fraction from a young rat contains only a small amount of intra- and intermolecularly linked collagen components (Fig. 7); together with the chemical data this suggests that the ISC_R fraction of a young rat consists of protein-polysaccharide complexes to which collagen components are bound to form an insoluble matrix. It is known also from other work that the ratio of hexosamine to collagen decreases in tissues during ageing (Sobel *et al.* 1953, Nimni *et al.* 1966). The presence of collagen in protein-polysaccharide complexes in shark cartilage has been demonstrated recently (Mashburn and Hoffmann 1967). The tentative model of Jackson and Bentley (1968) also takes glycosaminoglycans and mucoproteins into consideration in the formation of insoluble collagenous matrices.

In conclusion, it can be stated that the amount of insoluble collagen is largest in the skins of young animals where collagen molecules are distributed in a matrix of carbohydrates and non-collagenous proteins. The insolubility is due mainly to the interaction of collagen with other constituents of the extracellular space. The insoluble collagen of an adult rat is characterized by a mutual interaction of peptide chains. The biphasic solubility of insoluble collagen extracted at stepwise rising temperatures indicates that lateral packing of the collagen molecules is maintained by both labile and stable bonds. It is not known whether the accumulation of the ISC_{40} fraction with age is a result of retarded transformation of the ISC_{40} fraction into the ISC_R fraction or diminished synthesis of other extracellular constituents which allows the association of the collagen components. The increase of the ISC_{40} fraction with age may be intimately connected with the ageing of collagen.

Metabolism of the collagen fractions

Turnover of the soluble collagens. The results presented in this study indicate that the turnover of 0.45 *M* NaCl soluble collagen is faster in a young than in an adult rat (Figs. 9 and 10). The half-life of collagen soluble in 0.45 *M* sodium chloride was 27 hours in 6-day-old rats and 73 hours in 3-month-old rats. These half-lives are shorter than those reported earlier (see review by Woessner 1968). It has been shown also by Kivirikko (1963) that collagen soluble in 1 *M* sodium chloride reaches its maximum

specific activity within 3 hours after isotope administration in chick embryos The use of the specific method for the determination of radio active hydroxyproline instead of the determination of radioactive glycine and the use of very young animals in the present study possibly explain the discrepancy

The slow initial incorporation of proline into the skin of an adult rat may result from a retarded synthesis or from environmental conditions such as a decreased flow of blood to the skin Also the activity of the system hydroxylating protocollagen decreases in the skin of the rat with age (Juva and Heikkinen 1968) this shows that various stages of collagen metabolism slow down with age

The results presented in Fig 11 show that the metabolism of collagen is faster in the skin of young rats also at the level of collagen components The rapid initial labelling of the α, β and γ components suggests that also an intracellular synthesis of β and γ components occurs The slow turnover of the β and γ components indicates that these components are formed slowly from the primary components the α chains also in the extra cellular space It has been suggested (Lapiere and Nusgens 1968) that all three components are released from the cells at specific rates and that each of them has a different turnover rate

The observed difference in the labelling of the $\alpha 1$ and $\alpha 2$ components suggests that besides certain structural differences (Hollmen and Kulonen 1964 Piez 1965) differences may also exist in the metabolism of the three chains of the tropocollagen molecule Slight degradation of collagen components during the gel electrophoretic run is not however excluded

Conversion of soluble collagens to more stable forms Transformation of 0 45 M NaCl soluble collagen into 0 5 M acetic acid soluble and insoluble forms occurs faster in the skin of a young rat than in the skin of an adult rat (Fig 9 and Table IX) The metabolic relationship between acetic acid soluble and insoluble collagens is not clear Conversion of 0 45 M NaCl soluble collagen to acetic acid soluble collagen probably involves the formation of stable cross links between the α chains Collagen molecules are then slowly linked together by intermolecular bonds Formation of insoluble collagen in the skin may occur by different mechanisms which are related to the age of animal The fast transformation of the 0 45 M NaCl soluble collagen into the insoluble form in the skin of a young rat may be connected with the rapid metabolism of carbohydrates and non collagenous proteins

Maturation and ageing of rat skin collagen Both the maturation and ageing of collagen are believed to involve the formation of linkages be

tween the peptide chains of collagen. The term 'maturation' is used to denote the orientation of the tropocollagen molecules into fibrillar arrangements in the extracellular matrix. The 'ageing' of collagen in contrast to maturation is something negative involving a slow deterioration of the collagenous structures with age. It is not yet known to what extent the biological properties of collagen are influenced by an increase in the number interchain linkages but it seems likely that the primary changes occur in the connective tissue cells and that the reactions of collagen molecules in the extracellular space are regulated by the changes in the cells.

Formation of insoluble collagen *in vitro*

General

Studies on the formation of insoluble collagen *in vitro* have usually been based on the precipitation of native type fibrils from collagen solutions and on observations of the changes in the solubility of fibrils or changes in collagen solutions incubated or stored under different conditions for varying periods of time. Knowledge of the formation of insoluble collagen in living cells *in vitro* is meagre. It can be stated on the basis of current knowledge that a variety of different agents augment fibril formation *in vitro*, but the relation of the results to the biological situation has not been clarified (see *e.g.* Wood 1964, Jackson and Bentley 1968).

Metabolic involvements

It has been suggested recently by Tsurufuji and Ogata (1965) that aerobic cellular activity is necessary for the formation of insoluble collagen. The results are not, however, conclusive.

In the present study the incorporation of labelled proline into collagen was followed *in vivo* and the transformation of the labelled 0.45 M NaCl soluble collagen into the insoluble form was studied during subsequent incubation of fresh radioactive skin slices *in vitro* (Fig. 12, Table X). The formation of insoluble collagen was not affected by anaerobic conditions, by an inhibition of the tricarboxylic acid cycle by sodium fluoride or by inhibition of glycolysis by sodium iodoacetate. Insoluble collagen was formed also in skin homogenates (Table XVII).

The formation [illegible]nsoluble collagen was slowest between pH 5.4 and 6.4 and increased [illegible] both acidic and alkaline directions in citrate phosphate (Fig. 14) and [illegible] acetate buffers. According to Wood and Keech

(1960) fibrils precipitate from collagen solutions at the lowest rate between pH 7 1 and 7 5 Vanamee and Porter (1951) observed the optimum pH for fibril formation in a solution of collagen in a citrate phosphate buffer to be between 6 0 and 7 5 The isoelectric point of raw, unpurified collagen was reported to be pH 5 8 by Cassel and Kanagy (1949)

An exposure of reactive groups which are responsible for the increased insolubility probably occurs on both sides of the isoelectric point No indisputable explanation of the effect of pH can however be given at this stage

Recent evidence supports the idea of a spontaneous cross linking of rat tail tendon collagen (Gross 1961) The thermal shrinkage of native rat tail tendon collagen fibres stored in 0 9 % sodium chloride suggested an increase in both intra and intermolecular cross linking (Rigby 1967) Metal catalysed and oxidative cross linking of rat tail tendon collagen was observed by Bello and Bello (1967) Evidence pointing to the existence of Schiff bases as labile intermolecular cross links in collagen has been presented (Bailey 1966) These labile cross links are gradually stabilized *in vivo* and on storage of the fibres in a buffer solution

The results obtained in the present study suggest that the formation of reactive groups involved in one type of cross link formation in collagen molecules is controlled by cellular activities (Table XX)

Effect of metal cations

The data presented in Figures 14 and 15 indicate that calcium ions in physiological concentration augment the formation of insoluble collagen in skin slices *in vitro* The binding of calcium ions to insoluble collagen during incubation (Table XIII) suggests that calcium ions play a direct part in the stabilization of collagen Recently also Steven (1967) presented evidence that calcium ions are involved in the interaction of collagen fibrils and the intercellular matrix in rat tail tendon The amount of calcium has been reported to be higher in acetic acid soluble and insoluble collagen fractions of chick embryo than in the 1 0 *M* NaCl soluble collagen (Naber *et al* 1967) this further supports the idea of metal mediated linkages in collagen The mechanism of the action of calcium is still obscure

Bello and Bello (1967) demonstrated that the formation of cross links in collagen depends on metal and oxygen concentrations when solutions of rat tail tendon collagen are incubated under different conditions Under anaerobic conditions and in the presence of chelating agents the in

solubilization is retarded It was further concluded that metal ions may act as oxidants in the early cross linking and that oxygen is involved mainly in the slower cross linking lasting several weeks

Carboxyl groups have suggested to be responsible for the binding of calcium to collagen (Gilbert 1960 Iron and Perkins 1962) In the present study calcium ions were found to augment the formation of insoluble collagen similarly on both the alkaline and acidic sides of the isoelectric point of collagen, this suggests that carboxyl groups are not alone involved in the formation of insoluble collagen under the employed conditions

It is well known from the leather industry that metal ions aggregate gelatin and collagen The idea of the physiological importance of metal cations is however new Adam and Kuhn (1968) have recently suggested that different metal cations e g gold, bismuth iron and copper, are able to introduce cross links between collagen molecules both *in vivo* and *in vitro* This would explain the effect of gold therapy in rheumatic diseases In the present study calcium ions could be replaced by a number of other metal cations (Tables XIV—XV), cobalt(II), copper(II) copper(I) and gold(III) were the most effective All of these metals have proved to be beneficial in the treatment of certain rheumatic diseases

Role of aldehyde groups

The tropocollagen molecules that dissolved in 0 45 *M* sodium chloride and 0 5 *M* acetic acid contained 2—4 acetaldehyde equivalents of reactive aldehyde groups (Table VIII) This is in good agreement with the results of Gallop (1964) Rojkind *et al* (1964) and Tanzer *et al* (1966) The specificity of the colour reactions used in the determinations of aldehyde groups is not known in full detail Non collagenous materials which contain carbonyl groups can also react with the colour reagents and invalidate comparison of the results of different authors The physiological functions of the aldehyde groups have usually been associated with the formation of cross links between chains in the tropocollagen molecules (Levene 1962) This concept is principally based on the observation that the collagen of lathyritic animals is deficient in intramolecular cross links (Martin *et al* 1961 Nikkari and Kulonen 1962) and that the relative number of aldehyde groups is proportionally reduced (Rojkind and Juarez 1966) According to the present study the contents of aldehyde groups in both 0 45 *M* NaCl and 0 5 *M* acetic acid soluble collagens of rat skin were reduced by administration of aminoacetonitrile and semicarbazide in the diet

The reduction of aldehyde group content was most apparent in the

α and β components of collagen The average number of acetaldehyde equivalents per tropocollagen molecule was 1 68 in the α components and 1 86 in the β components in the acid soluble collagen from a rat that received semicarbazide and 4 26 in the α components and 3 39 in the β components in the acid soluble collagen from a normal rat (Table VII) The average number of acetaldehyde equivalents per tropocollagen molecule was approximately the same in the γ components of both collagens (2 55 and 2 70)

Phenylhydrazine and semicarbazide inhibited the formation of insoluble collagen also *in vitro* (Fig 19) The effects could be explained by an inhibition of the aldehyde functions of collagen as suggested by Wood (1963) and Tanzer *et al* (1966) On the other hand semicarbazide and phenylhydrazine can also act as chelating agents This would explain the observed partial reversion of lathyrism produced by these agents when calcium salts are administered in excess (Naber *et al* 1967)

The metabolism of aldehyde groups in the formation of cross links has not been investigated Bornstein *et al* (1966) have stated that the lysine side chain of collagen can be converted to a grouping identified as the δ semialdehyde of α aminoadipic acid Two of these groups in separate chains in tropocollagen may be joined by a cross link possibly by aldol condensation Inhibition of the formation of the specific lysyl derivative is one possible property of certain lathyrogens as suggested by Page and Benditt (1967 1968)

The results presented in Table XX indicate that the formation of one type of aldehyde group is dependent on the cellular activities The subsequent events in cross link formation might be the formation of a Schiff base which is converted further to a stable intermolecular cross link with age as suggested by Bailey (1968) and Tanzer (1968) These reactions could occur nonenzymatically in the extracellular space Additional studies are necessary to confirm this suggestion and to define the role of aldehyde groups in cross link formation

Considerable experimental data support the significance of aldehyde groups in the formation of intramolecular cross links It was found (Table VI) that insoluble collagen which can be brought into solution only after degradation of the primary structure contains one aldehyde group less per tropocollagen molecule than the ISC_4 fraction This suggests that also intermolecular cross links may involve an aldehyde function

SUMMARY

The formation of insoluble collagen in rat skin was studied both *in vivo* and *in vitro* in order to clarify the mechanism by which tropocollagen units are transformed into supermolecular structures especially in relation to the ageing process

I Suitable methods were developed for the fractionation and purification of soluble collagens (Fig 1), for the determination of the specific radioactivity of hydroxyproline in starch gel electrophoretic collagen fractions (Table IV, Fig 2) and for the production of insoluble collagen *in vitro* (Fig 12)

II The proportion of insoluble collagen was maximal in the skin of a newborn rat The rapid growth period of the rat was characterized by an accumulation of collagen soluble in 0 5 M acetic acid at 4° (Fig 3) The insoluble collagen was separated into two subfractions (designated ISC_{40} and ISC_R) The proportion of collagen soluble in 0 5 M acetic acid upon heating at 40° (ISC_{40}) increased with age (Figs 4—5) ISC_R could be brought into solution only after degradation of the collagen at temperatures above 60° (Fig 5) Also the proportion of ISC_R was maximal in the skin of a newborn rat (Fig 4)

The subfractions of insoluble collagen extracted by 0 5 M acetic acid at 40° from skins of 5 day old rats consisted mainly of α and β components The proportions of β and γ components were higher in corresponding fractions from skins of adult rats (Fig 7) The ratio of hexosamine to hydroxyproline decreased rapidly in the insoluble collagen with advancing age (Table V)

The content of free aldehyde groups was lower in the collagen of a lathyritic rat than in the collagen of a normal rat The difference was largest in the α components (Table VII) The subfractions of insoluble collagen had different contents of aldehyde groups $ISC_{60\ 80}$ contained one acetaldehyde equivalent TC less than $ISC_{40\ 50}$ (Table VI)

Incorporation studies indicated that insoluble collagen is formed faster in young than in adult rats (Table IX Fig 9) The half lives of 0 5 M

NaCl soluble collagens from 6 day old and 3 month old rats were 27 and 73 hours respectively (Fig 10) The turnover of the $\alpha 2$ component in 0 1 M acetic acid soluble collagen from a 6 day old rat was faster than that of the $\alpha 1$ component (Fig 11)

III When skin slices labelled *in vivo* were incubated *in vitro* the total and specific radioactivities of the hydroxyproline of the insoluble collagen increased during 8 hours (Table X Fig 12) The formation of insoluble collagen during incubation was augmented by (1) a rise in temperature of the incubation medium up to 37° (Fig 13) (2) a decrease in the thickness of the slices to 0 3 mm (Table XI) and (3) the presence of calcium ions up to 3 0 mM concentration (Fig 15) The ^{45}Ca activity increased in the insoluble residue of skin when skin slices labelled *in vivo* were incubated *in vitro* (Table XIII)

Metal cations influenced the formation of insoluble collagen in the following order of decreasing effectiveness (Table XIV and XV) Co^{+} > Cu^{+} > Cu > Au^{3} = Ni > Fe^{3} > Hg > Cr^{3+} > Ca^{2+} > Fe > Al^{3} > Bb^{4} > Sn^{4+} > Sr = Ba^{2+} > Mn^{2} = Mg

The addition of EDTA and substances reacting with aldehyde groups (phenylhydrazine and semicarbazide) to the incubation medium (Figs 16—17) inhibited the formation of insoluble collagen

The rate of formation of insoluble collagen was lowest near pH 6 4 and increased in both alkaline and acid directions (Fig 14)

Attempts to influence the formation of insoluble collagen *in vitro* by employing anaerobic conditions and by adding sodium fluoride sodium iodoacetate *αα* dipyridyl and puromycin failed

The aldehyde groups in the 0 45 M NaCl soluble collagen increased when skin slices were incubated in the presence of glucose *in vitro* (Table XX)

Insoluble collagen was formed also when skin homogenates were incubated *in vitro* (Table XVII)

Two possible age dependent mechanisms leading to the formation of insoluble collagen are suggested The roles of metal cations and aldehyde groups in the stabilization of collagenous structures are discussed

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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENT 319

STUDIES ON TISSUE THROMBOPLASTIN, THROMBIN AND FIBRINOPEPTIDE IN INTRAVASCULAR COAGULATION

BY

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STUDIES ON TISSUE THROMBOPLASTIN, THROMBIN AND FIBRINOPEPTIDES IN INTRAVASCULAR COAGULATION

BY

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The present dissertation is a summary and discussion of the following papers

I Teger Nilsson A C A procoagulant from human postpartum serum I Assay and characterization *Scand J clin Lab Invest* 1967 *20* 329—338

II Teger Nilsson A C A procoagulant from human postpartum serum II Isolation and properties *Scand J clin Lab Invest* 1967 *20* 339—346

III Nordstrom S P Olsson A C Teger Nilsson and E Zetterqvist A pro coagulant from human postpartum serum Effects on coagulation and haemodynamics in dogs *Acta physiol scand* In press

IV Delin A P Olsson and A C Teger Nilsson Vasodilatation in the canine leg caused by intra arterial infusion of thrombin and tissue thromboplastin *Cardiovascular Research* 1967 *1* 371—378

V Olsson P J Swedenborg and A C Teger Nilsson Vasodilating and hy potensive effect of thrombin in dogs Role of fibrinogen plasma kinins and blood cells *Cardiovascular Research* In press

VI Teger Nilsson A C Degradation of human fibrinopeptides A and B in human serum *in vitro* *Acta chem scand* In press

VII Grondahl N J S P Miller and A C Teger Nilsson Isolation of fibrinopeptides formed during intravascular coagulation in dogs *Acta phy siol scand* In press

References to these papers will be made in the text by the Roman numerals listed above

CONTENTS

INTRODUCTION

Blood coagulation is accomplished through a series of biophysical and biochemical reactions involving several coagulation factors and finally resulting in the formation of fibrin threads Some of the reactions are well understood whereas others have been less studied To date thirteen coagulation factors have been described Most of them are normally present in plasma and some will under certain conditions be involved in a stepwise process leading to thrombin formation Extracts of tissues trigger this process The active principle in the tissues has been named tissue thromboplastin The pathways for thrombin formation with plasma factors alone (intrinsic coagulation) and plasma factors together with tissue thromboplastin (extrinsic coagulation) are partly separate Thrombin is a proteolytic enzyme which activates fibrinogen by release of the fibrinopeptides from the N terminal part of the molecule The activated fibrinogen then aggregates to form fibrin threads (For reviews on blood coagulation see Seegers 1967 Esnouf and Macfarlane 1968)

According to current views intravascular coagulation may occur with or without local thrombosis Coagulation without thrombosis is regarded as a continuous fibrin formation compensated for by a fibrinolytic process and by the ability of the reticuloendothelial system to engulf fibrin particles Several coagulation factors are consumed during intravascular coagulation In states of severe disseminated intravascular coagulation the blood concentration of fibrinogen and other coagulation factors may decrease considerably which in turn renders the blood incoagulable and may give rise to bleeding (For reviews on intravascular coagulation see Penick and Roberts 1964 McKay 1965 Rodriguez Erdmann 1965 Selye 1966)

Intravascular coagulation can be induced by several agents One of the most potent agents seems to be tissue thromboplastin which may enter the blood stream in connexion with certain traumata The placenta is particularly rich in tissue thromboplastin and placental complications during late pregnancy and delivery are believed to be an important cause of intravascular coagulation (Schneider 1950 a McKay 1965)

The main symptoms of intravascular coagulation are hypotension or shock bleeding and diffuse fibrin deposition in one or several organs with concomitant infarction and insufficiency Hypotension is an early symptom and may precede the bleeding The problems of shock and intravascular coagulation have been extensively studied (Hardaway 1966) but less attention has been focused on the haemodynamic effects of the separate coagulation factors and products

The physiological effects of the fibrinopeptides have been studied mainly *in vitro* Some authors have found that certain of them stimulate smooth muscles and have raised the question whether the fibrinopeptides may play a role in the microcirculation of the blood (Gladner 1966) Before such a question can be fully answered more must be known about release of the fibrinopeptides during coagulation *in vivo*, and about the fate of the fibrinopeptides after their release from the fibrinogen molecule

The present investigation deals with widely differing aspects of intravascular coagulation ranging from the trigger tissue thromboplastin to the products fibrinopeptides

A procoagulant from human postpartum blood was purified and characterized It had coagulation properties as well as some chemical properties and certain biological effects common to tissue thromboplastin

The effect of tissue thromboplastin and thrombin on haemodynamics in dogs was studied Thrombin but not tissue thromboplastin showed a peripheral vasodilating activity As thrombin is a key substance in all kinds of coagulation it was studied in detail and some possible mechanisms for its vasodilating effect were investigated

One of the physiological roles of thrombin is to initiate fibrin formation by release of the fibrinopeptides As the fibrinopeptides might play a role in the blood flow regulation and moreover be of value in the diagnosis of intravascular coagulation an investigation was undertaken on the fate of the fibrinopeptides in intravascular coagulation It was shown that fibrinopeptides can be isolated from blood after experimentally induced intravascular coagulation in dogs and that they probably are degraded in blood after their release from fibrinogen The fibrinopeptides had no effect on the peripheral blood flow in the system used in the present investigation

TISSUE THROMBOPLASTIN FROM HUMAN POSTPARTUM SERUM (PPS)

Previous investigations on tissue thromboplastin

In the middle of the 19th century it was observed that certain animal tissues showed a coagulation promoting activity Such an activity was first mentioned by Buchanan (1845) The coagulation promoting activity in tissue extracts was then studied extensively in particular with respect to its chemical and physical properties its mechanism of action in blood coagulation and its effects *in vivo*

Chemical and physical properties

The nature of the tissue accelerator was early investigated by several authors Wooldridge (1886) regarded it as a compound of phospholipids and protein Schmidt (1892) recognized that alcoholic extracts of tissues accelerated clotting Bordet and Delange (1913) McLean (1916) and others considered phospholipids to be the active material in the alcoholic extracts Morawitz (1904) showed that aqueous extracts of tissues had greater activity than alcoholic extracts and Howell (1912) like Wooldridge suggested that the active lipid was normally associated with a protein

In recent years more detailed investigations have been made on the composition of the active material in the tissues Tissue thromboplastin from lung brain and placenta has been most studied

Chargaff and co workers purified an extract from bovine lungs by means of high speed centrifugation and studied the product by ultracentrifugation electrophoresis and electron microscopy They stated it to be a lipoprotein of particulate matter with a particle weight of 167×10⁶ (Cohen and Chargaff 1940, 1941 a b Chargaff *et al* 1942 Chargaff and Bendich 1944 Chargaff *et al* 1944) The activity of bovine lung microsomes in blood coagulation was studied by Williams (1964) who found the microsomes to have a high coagulation promoting activity dependent on the presence of calcium ions and phospholipids

Hecht Cho and Seegers (1958) isolated a highly active material from rabbit brain This material contained no protein but consisted only of lipids The authors referred to it as brain thromboplastin Irsigler (1964) and Deutsch *et al* (1961) purified a thromboplastic fraction from human brain They stated it to be a lipoprotein and separated the lipid from the lipoprotein complex The lipid as well as the protein moiety were further characterized Irsigler *et al* (1965)

reported that the earlier known species specificity of tissue thromboplastin was determined by the protein Hvatum and Prydz (1966) released the thromboplastic activity from the microsomal fraction of human brain by treatment with deoxy cholate obtained further purification by gel filtration and achieved reaggregation on removal of the deoxycholate

Clarke and O Meara (1966) investigated the microsomal fraction of human chorionic cells and found a particulate material that accelerated blood clotting even in high dilutions Williams (1966) reported on the isolation and coagula tion activity of both human placenta microsomes and brain particles

Mechanism of action

A large number of hypotheses on the mechanism of action of tissue thrombo plastin in blood coagulation have been presented Of the early investigators in this field mention can be made of Morawitz (1904 1905) and Fuld and Spiro (1904) who expressed the view that the tissue substance acted as a kinase to initiate and hasten the formation of thrombin from prothrombin and calcium in plasma

Later Biggs *et al* (1953) Flynn and Coon (1953) and Hardisty (1955) studied the reactions between brain extracts, factor V and factor VII They found that these three agents took part in a reaction producing a prothrombin converting principle The reaction between tissue thromboplastin and factor VII was extensively studied by Hjort (1957) who postulated the formation of a reaction product between factor VII tissue thromboplastin and calcium Hougie (1959) studied the reaction between factor X brain extracts and factor V Straub and Duckert (1961) postulated that tissue factor and factor X react together in the presence of factor VII and calcium ions to form the so called extrinsic reaction product The extrinsic reaction product then reacts with factor V to form the extrinsic prothrombin activator which in turn converts prothrombin to thrombin Nemerson and Spaet (1964) found that brain thromboplastin contains two separable clotting activities one activated factor X and the other supplied phosphatide Josso and Prou Wartelle (1965) studied the interaction of tissue factor and factor VII and postulated that their reaction product might have the same role in blood coagulation as the reaction product of factor XI factor XII and glass Nemerson (1966) postulated a rapid reaction between tissue thrombo plastin and factor VII The formed product was tightly bound to tissue particles and could activate factor X The phospholipid requirement of tissue thrombo plastin was recently studied by Nemerson (1968)

It was early noticed that the coagulation promoting activity of tissues was inhibited by serum and plasma (Loeb 1904) Many investigators subsequently reported on inhibitors of tissue thromboplastin The work on the natural inhibitors

of tissue thromboplastin was reviewed by Hjort (1961) In addition to the inhibitors normally present in blood other agents *e g* certain phospholipids (Hecht 1965) and the bacterial enzyme thromboplastinase (Gollub *et al* 1953 Kushner and Feldman 1958) have an inhibiting or destructive effect on tissue thromboplastin

In vivo reactions

Attention was early focused by several authors on the effect of tissue extracts on the blood *in vivo* Thus Wooldridge (1886) found that intravenous injection of tissue extracts in animals gave rise to intravascular thrombus formation Gutmann (1914) and Mills (1921) reported that such injections rendered the blood incoagulable due to a decrease in fibrinogen concentration Ratnoff and Conley (1951) infused homologous brain thromboplastin into dogs and studied the induced coagulation defects Penick *et al* (1958) found that tissue thromboplastin from lung triggers both the extrinsic and intrinsic clotting mechanisms and Lewis and Szeto (1962) identified an anticoagul nt in blood after infusion of brain thromboplastin (For review on the effect of tissue thromboplastin *in vivo*, see Penick and Roberts 1964)

A connexion between postpartum haemorrhage and premature separation of the placenta was early observed (De Lee 1901 Williams 1915 Willson 1922) In view of the probable role of placental compounds in the pathogenesis of these conditions placental extracts have been widely used in animal experiments The activity of placental extracts *in vivo* was first studied by Obata (1919) Sakurai (1929) called attentation to the fact that placental toxins were similar to tissue coagulants Copley (1945) studied the effect of placental extracts on coagulation *in vivo* and *in vitro* Schneider (1947) identified the toxic principle of these extracts as tissue thromboplastin Some investigators infused placental extract into animals and reproduced the defibrination syndrome found in delivery (Fulton and Page 1948 Schneider 1950 b) Schneider (1950 a) and Page *et al* (1951) suggested that tissue thromboplastin from placenta may escape into the maternal circulation giving rise to fibrinogen consumption fibrin formation and concomitant incoagulable blood

Once these fundamental animal experiments had been performed, and the connexion between the thromboplastic activity of placental extracts and abnormal postpartum haemorrhage had been recognized many investigations were made on the defibrination syndrome in obstetrical cases Opinions still vary regarding whether intravascular coagulation with secondary fibrinolysis or primary fibrinolysis is the main reason for the defibrination These problems were reviewed by Schneider (1959) Zilliacus (1960) Stamm (1962) McKay (1965) Scott (1968) and others

Cohn s fraction I from human postpartum serum has been shown to have a coagulation promoting activity (Herrlin and Thilen 1955 Blomback and Blomback 1961) The nature of this procoagulant was investigated since knowledge about it might be of importance for the understanding of coagulation disorders in late pregnancy Purification of the procoagulant was started and an assay method was worked out The coagulation properties some chemical properties and some biological effects of the procoagulant were studied

Assay

The procoagulant from human postpartum serum (PPS) was active in several coagulation assay systems and also showed some esterase activity (I) The most convenient assay for purification and characterization seemed to be the two stage system with partially purified coagulation factors described in Paper I In this system PPS solution phospholipid reagent factor V reagent prothrombin reagent and calcium ions were incubated resulting in thrombin formation The thrombin formation was interrupted by addition of EDTA After a certain incubation time the amount of thrombin formed was proportional to the amount of PPS added *provided that the other conditions of the test were constant* The amount of thrombin formed was estimated by measuring the clotting time of purified fibrinogen and was expressed in NIH units by interpolation on a thrombin fibrinogen standard curve The activity of PPS was expressed in PPS units one unit being the amount of PPS that generated one NIH unit of thrombin in 3 minutes under the conditions of the test Concentrations of PPS corresponding to as little as about 2 μg protein/ml could be determined with this method The reproducibility was sufficient for purification purposes The different reagents could be excluded separately and the requirements of PPS for accessory factors thus investigated

The influence of ionic strength pH and buffer concentration on the apparent avtivity of PPS in the assay was investigated (I) The system showed a high sensitivity to changes in ionic strength above 0 1 Imidazole also had a remarkable inhibitory effect

The influence of variable concentrations of the reagents was studied (I) It was possible to find concentrations of phospholipid reagent factor V reagent and calcium ions which were optimal in the assay The optimal concentration expressed as μg P/ml reagent was the same for phospholipids from platelets as from soy beans The apparent activity of PPS was however directly proportional to the concentration of the prothrombin reagent The use of accurate amounts of prothrombin reagent was therefore of extreme importance Contamination of the

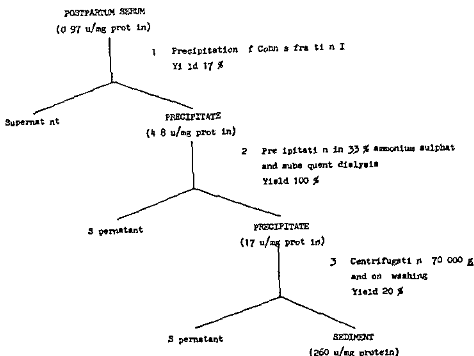


Fig 1
Purification procedure of PPS

samples to be assayed by prothrombin and prothrombin like factors could give erroneously high PPS activity Such contaminations were however of minor importance as shown in Paper II

Purification

PPS was purified in three steps (1) fractionation with ethanol in the cold according to Cohn (Cohn *et al* 1946) (2) precipitation in ammonium sulphate and subsequent dialysis and (3) high speed centrifugation (II) The purification procedure, specific activities and yields are shown in Fig 1

Fractionation according to Cohn was as a rule made on a large scale by a pharmaceutical firm (Kabi AB) as routine in their production of human serum proteins No attempts were therefore made to improve the yield and purification of PPS in this step

The yield of the second step was high possibly due to removal of inhibitors of PPS activity during fractionation Since dialysis was necessary after precipitation with ammonium sulphate the purification and yield of these two procedures are accounted for together

Good purification was achieved by means of high speed centrifugation and subsequent washing The yield in this step was however fairly low and it did not improve with the varying centrifugation conditions tested

Some other methods for purification were tried without success Gel filtration and chromatography did not result in any separation PPS did not move on paper or in agar gel during electrophoresis Detergent (sodium lauryl sulphate) had in my hands a deleterious effect on PPS activity

Procoagulant from human normal serum

The findings of Herrlin and Thilen (1955) indicate that PPS should be found only in postpartum serum A preparation of PPS from normal and postpartum serum was therefore made in parallel PPS from postpartum serum was about 10 000 times as active as PPS from normal serum (II) The slight activity found in unfractionated normal serum may be accounted for by the sensitivity of the assay to the serum factors VII and X and possibly also by the presence of small amounts of prothrombin The results show that the purified PPS is not contaminated by major amounts of coagulation factors from the blood Thus PPS is not present in normal blood but is associated with pregnancy

Properties of the purified PPS

Coagulation properties The possible contamination of PPS by other coagulation factors was also examined by modifications of the assay system (I) When PPS was incubated in the solvents of the first step of the assay and then added to fibrinogen no clotting occurred for at least 1 hour This indicates that PPS is not contaminated with thrombin nor with other coagulation factors in concentrations high enough for thrombin formation

The activity of PPS was also measured after exclusion of the different reagents from the assay (I) These experiments showed that the presence of factor V reagent and prothrombin reagent was necessary for the activity of PPS Although the presence of phospholipid reagent was not a prerequisite addition of phospholipids increased the activity

The relations of PPS to factors VII VIII IX and X were examined in a separate experiment whose principle was to substitute different deficiency plasmas for the prothrombin reagent (I) As expected from others and own observations the thrombin formation with factor VIII and IX deficient plasmas w s almost similar to the thrombin formation with normal plasma The thrombin formation with factors VII and X deficient plasma was however much slower This indicates that the presence of factors VII and X is necessary for the activity of PPS whereas the presence of factors VIII and IX is not

In conclusion it can be stated that PPS does not contain appreciable amounts of other coagulation factors and that it acts as a procoagulant in the presence of factors V, VII and X and calcium ions Phospholipid increases its activity

Stability The purified PPS was fairly stable during storage It could be heated to 50 ° C without loss of activity and it was stable in the pH range 3—11 It did however lose some activity when lyophilized (II) The activity of PPS resisted treatment with diisopropylfluorophosphate indicating that the activity was not connected to thrombin or trypsin like enzymes On the other hand the activity decreased considerably during incubation with a heated snake venom containing phospholipase A and it was not possible to regain the activity by addition of further phospholipids This indicates that intact phospholipids perhaps in membrane structures are necessary for the PPS activity

Chemical composition Determination of N terminal amino acids showed that several peptide chains were present in the PPS preparations The most abundant N terminal amino acids were glutamic acid glycine aspartic acid alanine valine and leucine/isoleucine The results indicate that PPS is heterogeneous with respect to its protein component

For comparison with certain tissue thromboplastins analyses of nitrogen phosphorus and carbohydrates were made The average phosphorus/nitrogen ratio calculated on a molar basis was 1 44 This ratio has been used by other investigators as an indication of the proportions of phospholipid and protein in their preparations The findings for PPS were comparable to the finding of Deutsch *et al* (1964) for purified human brain thromboplastin The bovine lung thromboplastin described by Chargaff *et al* (1942) had a somewhat higher proportion of phosphorus than PPS The concentration of phosphorus was higher and that of carbohydrates lower than that reported by Aoki and von Kaulla (1966) for a thromboplastin like procoagulant isolated from human urine

In vivo reactions Tissue thromboplastin is known to induce intravascular coagulation Consumption of coagulation factors and hypotension are the main early features of this condition Consequently the blood concentration of some coagulation factors *i e* fibrinogen fibrinogen labelled with radioactive iodine prothrombin and platelets was measured and the blood pressure recorded after injections of PPS into a central vein in dogs (III) In addition the blood flow in the extremities was studied after local intra arterial infusion of PPS (III)

Intravenous injection of PPS induced a decrease in the concentration of fibrinogen labelled fibrinogen prothrombin and platelets indicating a consumption of these factors Furthermore it brought about a fall in blood pressure PPS had however no effect on the leg blood flow on local intra arterial injection The findings suggest that PPS when injected intravenously gives rise to intravascular coagula

tion The hypotension is probably not due to contamination of PPS by vasoactive material but may at least partially be secondary to changes during the intra vascular coagulation process

Similarities between PPS and tissue thromboplastin

During the work with the procoagulant from postpartum serum it became increasingly evident that it behaved in many respects like a tissue thromboplastin although isolated from blood Some arguments for PPS being a tissue thrombo plastin are summarized in the following

Tissue thromboplastin is known to promote activation of prothrombin to thrombin in the presence of factors V VII and X (for references see page 10) The same was shown to apply to PPS Factor VII is besides tissue thromboplastin the only factor specific to the extrinsic coagulation system The requirements of PPS for factor VII are therefore of special significance in this connexion The sensitivity of PPS to a phospholipase containing snake venom resembled the sensitivity of tissue thromboplastin to another phospholipase the so called thromboplastinase (Gollub *et al* 1953 Kushner and Feldman 1958) The behavi our of PPS in solutions and during high speed centrifugation indicates that it consists of particulate matter Similar observations have been made by others on tissue thromboplastins from different sources (Chargaff *et al* 1942 Deutsch *et al* 1964 Williams 1964 Clarke and O Meara 1966 Hvatum and Prydz 1966) The analyses of phosphorus and nitrogen showed that in this respect PPS was comparable to the human brain thromboplastin purified by Deutsch *et al* (1964) When injected into animals PPS induced intravascular coagulation like tissue thromboplastins (Nordstrom and Zetterqvist in press)

Taken together these observations suggest that PPS is a tissue thromboplastin The absence of an activity similar to PPS in normal serum supports this view and indicates that it is related to pregnancy It is known that the placenta is rich in tissue thromboplastin (Copley 1945 Schneider 1947 Clarke and O Meara 1966 Williams 1966) It is thus probable that PPS is a tissue thromboplastin from placenta or related tissues released into the postpartum blood during delivery

Summary

A procoagulant from human postpartum serum was purified and characterized and it was shown that no such procoagulant is present in normal human serum

The procoagulant was assayed in a two stage coagulation system with partially purified coagulation factors It was purified with fractionation according to Cohn precipitation with ammonium sulphate and subsequent dialysis and with high speed centrifugation

The coagulation properties of the purified procoagulant were essentially the same as those of tissue thromboplastin thus it promoted activation of prothrombin to thrombin in the presence of factors V VII and X Although the presence of phospholipid was not a prerequisite it increased the activity The behaviour of the procoagulant during high speed centrifugation its sensitivity to phospholipase and some of its chemic l properties agreed with earlier findings for tissue thromboplastin Intravenous injection of the procoagulant into dogs induced a consumption of coagulation factors and hypotension interpreted as intravascular coagulation

It is concluded that the procoagulant is a tissue thromboplastin probably derived from placental tissues during delivery

HAEMODYNAMIC EFFECTS OF TISSUE THROMBOPLASTIN AND THROMBIN IN DOGS

Previous investigations on the vasoactivity of blood coagulation factors

Most investigators who have studied the problem of intravascular coagulation experimentally and clinically have noted haemodynamic changes in connexion with coagulation It is evident that intravascular thrombus formation results in circulatory disturbances Coagulation activities too low for clot formation might however also give rise to haemodynamic changes owing to formation or release of vasoactive material during the coagulation process Brodie (1903) supposed that substances which could excite the muscular walls of the blood vessels were produced during blood coagulation He also stated that the presence of blood cells was essential for formation of the vasoactive material (Brodie 1900) Freund (1920) found that autologous defibrinated blood produced systemic hypotension in some animals The early appearance of hypotension in states of disseminated intravascular coagulation (Hardaway 1966) indicates that vasoactive material might be formed or released during the process of coagulation Only few investigations on the vasoactivity of the separate coagulation factors or products have however been made It is mainly the role of some platelet factors the connexion between blood coagulation and vasoactive plasma kinins and the physiological role of the fibrinopeptides that have been studied

The role of platelet factors was investigated by Zucker and co workers who showed that thrombin releases serotonin a smooth muscle stimulant from blood platelets (Zucker and Borrelli 1955) Zucker (1959) expressed the view that the serotonin released during platelet plug formation played no important role in haemostasis Grette (1962) carried out detailed studies of the thrombin induced release reaction and found that thrombin releases serotonin and adenine nucleotides in large amounts from platelets The problem of serotonin and histamine release from platelets was also studied by Humphrey and Jaques (1955) Waalkes *et al* (1957) Gaintner *et al* (1962) and Markwardt and Barthel (1964) among others Groth (1966) and Groth *et al* (1966) infused thrombin into rabbits and interpreted a subsequent fall in blood pressure and oxygen tension as being at least partially due to release of serotonin and histamine from the platelets Haustein and Markwardt (1965) studied the influence of separate coagulation factors from human blood on the vascular bed of an isolated pig ear and found that thrombin exerted vasoconstriction via release of serotonin and ATP

from the platelets (For reviews of the biochemistry and function of platelets see Marcus and Zucker 1965 Johnson 1967)

Some associations between blood coagulation fibrinolysis and vasoactive plasma kinins have recently been studied Margolis pointed out that activated factor XII) besides initiating coagulation also initiates the release of vasoactive plasma kinins (Margolis 1960 Margolis 1963 Margolis and Bishop 1963) Back and co workers (Back *et al* 1963 a b Back and Steger 1965 Back 1966 Back *et al* 1966) studied the connexion between fibrinolysis and plasma kinins *in vivo* and *in vitro* and found that plasmin can release plasma kinins in at least two different ways Haustein and Markwardt (1965) found that plasmin affected the blood flow in a pig ear via activation of plasma kinins

The role of the fibrinopeptides in the haemodynamics of blood coagulation is at present the subject of much interest Some investigators have claimed that certain fibrinopeptides stimulate smooth muscles and have suggested that fibrino peptides may play a role in the microcirculation of the blood (For references see page 27)

Olsson and Delin (1965) showed that small amounts of thrombin and tissue thromboplastin when infused into the femoral artery of a dog gave an increase in blood flow in the artery due to vasodilatation whereas large amounts of these substances produced an initial increase followed by a decrease, probably due to coagulation

Present investigation

This study presents further and more detailed experiments on the peripheral vasodilating activity of tissue thromboplastin and thrombin In addition the effect of thrombin on blood pressure in defibrinogenated animals was studied An attempt was made to elucidate the mechanism of the vasodilating and hypotensive effects of thrombin The role of products of the thrombin fibrinogen reaction plasma kinins and substances from blood cells as mediators of the vasoactivity of thrombin was investigated

Experimental conditions

The experiments were made on anaesthetized dogs The animals were artificially ventilated with a respirator One or both femoral arteries and one carotid artery were exposed The test solutions were infused at a constant rate into one femoral artery and physiological saline for control into the other via catheters introduced through small arterial branches With some exceptions the infusion rate did not exceed 10 % of the blood flow in the artery The blood flow in the femoral arteries was measured proximal to the infusion sites by means of extra

vascular square wave electromagnetic flowmeters The blood pressure was as a rule measured in the aorta Femoral arterial blood flow and blood pressure were recorded continuously The concentrations of the test substances in the arterial blood were calculated assuming immediate and complete mixing

Effects of tissue thromboplastin on peripheral and general circulation

Infusion of homologous crude tissue thromboplastin from lung or brain into a femoral artery of the dogs gave different reactions depending on the dose (IV) Small amounts of tissue thromboplastin produced an increase in blood flow in the test leg but did not influence the flow in the contralateral leg or the blood pressure Larger amounts of tissue thromboplastin gave an initial increase in blood flow in the test leg followed by a fall in blood pressure and a subsequent decrease in blood flow both in the test leg and the contralateral leg Heparinization of the animal did not influence the local flow increasing effect of crude tissue thromboplastins but inhibited the fall in blood pressure and the subsequent decrease in peripheral blood flow

The haemodynamic effects of crude tissue thromboplastin are thus to be ascribed to at least two different entities One of them is sensitive to heparin and is probably equivalent to the coagulation trigger in tissue extracts An attempt was made to separate these entities by dialysis (IV) The vasoactive material but no procoagulant material appeared in the dialysate whereas all the procoagulant material as expected remained in the dialysis bag This result suggests that the local vasodilating activity of crude tissue thromboplastin is due to contamination by low molecular substances This conclusion is strengthened by the observation that purified tissue thromboplastin from human postpartum blood when administered intra arterially does not produce any increase in the local blood flow (III) The hypotensive effect which was demonstrated with larger amounts of the purified human tissue thromboplastin might be due in part to activation of the blood coagulation process (III)

Effects of thrombin on peripheral and general circulation

The formation of thrombin is inevitable in intravascular coagulation triggered by tissue thromboplastin or induced in other ways The study was therefore continued with experiment on the haemodynamic propertie of thrombin

Peripheral circulation Infusion of small amounts of thrombin into the femoral artery produced a considerable increase in blood flow (IV) There was an initial peak followed by a raised flow level of the same duration as that of the thrombin infusion On cessation of the infusion the blood flow rapidly returned to the pre infusion level (Fig 2 A) Infusion of large amounts of thrombin gave an initial peak in the flow after which it rapidly decreased to zero (Fig 2 B) Local

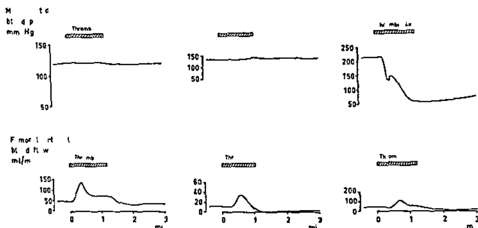


Fig 2

A Infusion of thrombin into a femoral artery giving a calculated local initial concentration of 0 4 NIH units/ml blood

B Infusion of thrombin into a femoral artery giving a calculated local initial concentration of 15 NIH units of thrombin/ml blood

C Infusion of 2500 NIH units of thrombin into a central vein of a defibrinogenated dog.

infusion of thrombin resulted as a rule in no changes in the blood pressure nor in the blood flow in the contralateral leg

The observed flow changes are probably the net result of at least two processes with opposite influences on the flow

The increase in blood flow cannot be explained by a local decrease in fibrinogen concentration with a subsequent decrease in blood viscosity (Wells *et al* 1964) Since the blood pressure and blood flow in the control leg were unchanged the increase in blood flow must have been an expression of a diminshed peripheral resistance depending on local vasodilatation The rectilinear dose response relation between the calculated intra arterial concentration of thrombin and the maximal blood flow found at low concentrations (IV) indicated that the vasodilating activity of thrombin dominated at these concentrations

The decrease in blood flow found at higher thrombin concentrations is probably due to platelet aggregation and fibrin formation *i e* coagulation although release of vasoconstrictor substances cannot be ruled out

General circulation In contrast to the finding with tissue thromboplastin thrombin produced no significant changes in blood pressure even after doses giving a local concentration of up to about one NIH unit/ml arterial blood (IV)

This indicates that thrombin or any hypothetical vasoactive products released by it are rapidly inactivated or sufficiently diluted in the blood

Attempts to increase the dose of thrombin to ascertain whether its vasodilating activity could give rise to general hypotension would necessarily be unsuccessful due to interference by clot formation It was however found that the peripheral vasodilating effect of thrombin was the same in normal animals and in defibrino genated animals unable to form intravascular clots (V) Defibrinogenated animals thus offered excellent objects for studies of the hypotensive effect of thrombin without interference of clot formation In such animals intravenous infusion of thrombin to a roughly estimated concentration of about one NIH unit/ml blood brought about a fall in blood pressure (Fig 2 C) It is thus probable that the observed local vasodilating effect of thrombin may be generalized and under certain circumstances give rise to hypotension

Purity of thrombin preparations Most observations on the vasoactivity of thrombin were made with a commercial thrombin with a comparatively low degree of purity It was therefore important to establish whether there occurred vasoactive impurities in the thrombin preparations (IV and V)

Heparinization of the animals completely abolished both the flow increase flow decrease and hypotension obtained after thrombin infusions as did treatment of the thrombin with diisopropylfluorophosphate The coagulation activity as well as the vasoactivity of thrombin persisted after dialysis A highly purified thrombin produced the same flow changes as the commercial one when diluted to equal coagulation activities These observations taken together strongly suggest that the vasoactivity of thrombin is correlated to its coagulation activity and that the observed effects are due to the enzyme thrombin and not to impurities in the preparations

Mechanism for the vasodilating effect of thrombin

Thrombin is a proteolytic enzyme with a narrow substrate specificity for fibrinogen It has however at least two different functions in the coagulation process *i e* it splits off fibrinopeptides from the fibrinogen molecule and it disintegrates platelets It is also known that thrombin in high concentrations may attack some other proteins than fibrinogen (Pantlitschko and Grundig 1957 Thelin and Wagner 1959 Mutt *et al* 1965 Engel *et al* 1966) In view of these facts some hypotheses on the mechanism for the vasoactivity of thrombin were investigated

Products of the thrombin fibrinogen reaction It has been claimed that some fibrinopeptides stimulate smooth muscles *in vitro* and physiological effects of the fibrinopeptides *in vivo* have been reported (Gladner 1966 Bayley *et al* 1967)

The role of the products of the thrombin fibrinogen reaction for the per i vasodilating activity of thrombin was therefore first studied

Purified canine fibrinopeptides were infused into the femoral arteries of to concentrations equivalent to those which might occur during fibrin for t i *in vivo* No changes in blood flow or blood pressure were observed durin c c infusions (IV) Clot supernatants from canine fibrinogen were likewi c inac i e when infused intra arterially (V) These findings indicate that the fibrinopep d s or other products of the thrombin fibrinogen reaction are unlikely to be mediators of the peripheral vasoactivity of thrombin The finding that defibrinogenated animals showed the same response to intra arterially infused thrombin as normal animals supports this view (V)

Role of plasma kinins Trypsin kallikrein bradykinin and thrombin all elicited the same type of response when infused intra arterially (IV) This observation and the fact that some relations between the blood coagulation system and plasma kinin releasing system have been described (Margolis 1963 Back 1966) lead to the supposition that plasma kinins might be mediators of the vasoactivity of thrombin Although the substrate specificity of thrombin is limited a direct or indirect release of plasma kinins by thrombin could not be excluded

Purified bovine bradykininogen was incubated with thrombin trypsin or kallikrein (V) The incubates of trypsin or kallikrein with bradykininogen showed vasoactivity when infused intra arterially in a heparinized dog This was expected since trypsin and kallikrein are known bradykinin releasing enzymes The incubates of thrombin and bradykininogen exhibited no vasoactivity even if large amounts of thrombin were used It is thus improbable that thrombin exerts its vasoactivity by release of bradykinin from bradykininogen

It is however possible that thrombin may attack and activate some other precursors than bradykinogen in the series of reactions leading to plasma kinin release Since these reactions are not clarified chemically this problem had to be investigated in a completely different way (V) Intravenous infusions of large amounts of kallikrein made dogs transiently resistant to intra arterial infusion of kallikrein and bradykinin During this resistant period the response to intra arterial infusion of thrombin was essentially unchanged Moreover rapid administration of large amounts of thrombin intravenously to defibrinogenated animals produced a brief resistance to intra arterial infusion of thrombin During this resistant period the response to intra arterial infusion of bradykinin and kallikrein was not diminished On the contrary an increased response to kallikrein was observed The increased sensitivity to kallikrein was also found after defibrinogenation alone It is therefore improbable that the vasodilatation brought about by thrombin is due to the same mechanism as that produced by bradykinin and kallikrein This implies that plasma kinins are unlikely to be mediators of the vasoactivity of thrombin

Role of the blood cells Neither fibrinopeptides nor some of the known plasma kinins seemed to be involved in the vasodilating activity of thrombin Thrombin does however induce a release of vasoactive material from platelets the most abundant being serotonin and adenine nucleotides (Grette 1962) Platelet disruption with release of vasoactive material might therefore be a mechanism for the vasoactivity of thrombin The finding that defibrinogenated animals with an associated low platelet count showed similar response to intra arterially infused thrombin as did normal animals was however somewhat contradictory to this hypothesis (V) As the effect of thrombin on erytrocytes and leukocytes is essentially unknown the experiment was planned so that the effect of these three types of cells was studied together

Blood was drawn from dogs and separated into platelet poor plasma and platelet rich cell suspension Whole blood cell suspension and plasma were incubated with thrombin *in vitro* By using low thrombin concentrations and short incubation times interfering clot formation was avoided The incubates were infused into the femoral arteries of heparinized animals Vasoactivity appeared during incubation of whole blood and thrombin This also applied to cell suspensions and thrombin Plasma and thrombin were on the other hand inactive The results show that not only fibrinopeptides and plasma kinins but also other components of plasma can be ruled out as mediators of the vasoactivity of thrombin The presence of blood cells seemed however to be of importance

It has later been shown by Olsson and co workers (in press) that in similar experiments thrombin released ADP or ATP from the blood cells and that these nucleotides are the main mediators of the peripheral vasodilating activity of thrombin

Comparative aspects of thrombin and tissue thromboplastin

Intra arterial infusions of thrombin gave rise to local vasodilatation probably due to the release of vasoactive material from the blood cells Intra arterial infusions of dialyzed or purified tissue thromboplastin had no flow increasing effect despite the ability of tissue thromboplastin to form thrombin in the blood (IV) These apparently contradictory findings are explained by the fact that thrombin formation in blood after addition of tissue thromboplastin is a relatively slow pro ess The circulation time from the infusion site in the artery to the re eptor organs in the vessels is roughly 3 seconds (Glasser 1964) and this time is probably too short for the formation of sufficient activities of thrombin Thus intra arterially infused purified tissue thromboplastin does not directly influence the local blood flow but if the concentrations are high enough thrombin may be formed on the venous side and vasodilatation and hypotension may occur secondarily

Formation of thrombin with a concomitant release of vasoactive material from blood cells is probably one of the possible mechanisms for the hypotension observed after infusion of purified tissue thromboplastin from human postpartum serum (III) The estimated decrease in prothrombin concentration showed that as much as 10—20 NIH units of thrombin/ml plasma might have been formed These concentrations are much above those necessary for the hypotensive effect of thrombin

The pathophysiological significanse of the observations presented is difficult to evaluate since the clinical states of disseminated intravascular coagulation are most probably different from and more complicated than the experimental intra vascular coagulation induced by tissue thromboplastin and thrombin It is never theless possible that thrombin in vasodilating and hypotensive concentrations may be present in the blood stream in such conditions despite a high antithrombin activity of plasma

Summary

Tissue thromboplastin and thrombin were infused into a femoral artery or into a central vein of dogs and the femoral arterial blood flow and the aortic blood pressure were registered

Intra arterial infusion of dialyzed homologous tissue thromboplastin or purified human tissue thromboplastin had no local flow increasing effects Larger amounts produced hypotension which might have been due partly to thrombin formation

Intra arterial infusion of small amounts of thrombin resulted in a local flow increase whereas larger amounts produced an initial increase rapidly followed by a decrease The increase in blood flow seemed to be due to vasodilatation whereas the decrease was probably due to coagulation Thrombin given intra venously to defibrinogenated animals caused a fall in the aortic blood pressure

Fibrinopeptides and clot supernatants from fibrinogen had no vasodilating activity The vasodilating activity of thrombin was the same in normal as in defibrinogenated animals It is thus improbable that products of the thrombin fibrinogen reaction are mediators of the thrombin induced vasodilatation

Large intravenous infusions of kallikrein made dogs resistant to intra arterially infused kallikrein and bradykinin but not to thrombin Large intravenous infu sions of thrombin on the other hand made the dogs resistant to intra arterially infused thrombin but not to kallikrein and bradykinin These results and the finding that no vasoactivity was generated during incubation of bradykininogen with thrombin *in vitro* indicate that the vasodilating activity of thrombin is not mediated by plasma kinins

No vasoactivity was found after incubation of platelet poor plasma with throm bin *in vitro* However incubates of whole blood or platelet rich cell suspension with thrombin showed vasoactivity The presence of blood cells thus seems to be of importance for the vasoactivity of thrombin

Previous investigations on fibrinopeptides

As early as 1876 Hammarsten claimed that a hydrolytic cleavage of the fibrinogen molecule occurred during the fibrinogen fibrin transition This concept was not however definitely proved until 1950 by the fundamental work of Lorand Bettelheim Bailey and Middlebrook These authors isolated and studied two peptides from thrombin clotted bovine fibrinogen by means of electrophoresis and partition chromatography and denoted them as fibrinopeptides A and B (Bettelheim and Bailey 1952 Lorand 1952 Lorand and Middlebrook 1953 Bailey and Bettelheim 1955 Bettelheim 1956) The same fibrinopeptides were later isolated by various chromatographic methods (Blomback and Vestermark 1958 Gladner *et al* 1959) Using the technique of ion exchange chromatography Blomback and co workers isolated fibrinopeptides from a great number of mammalian species (Blomback and Sjoquist 1960 Blomback *et al* 1965 a b) Fibrinopeptides from primitive vertebrates such as the lamprey and from lizard and birds have also been isolated and studied (Doolittle 1965 a b Hann 1966)

The amino acid sequence of bovine fibrinopeptides was worked out indepen dently in two laboratories (Blomback and Wallen 1959 Folk *et al* 1959 a b Sjoquist *et al* 1960) At that time however some uncertainty persisted about the N terminal amino acid of fibrinopeptide B Blombäck and Doolittle (1963 a) later showed the N terminal amino acid to be pyroglutamic acid The structures of the fibrinopeptides from more than 20 mammalian species are now known The structural similarities and differences have been proposed to reflect the phylogene tic relation between the species (Doolittle and Blomback 1964 Blomback *et al* 1965 c Blomback *et al* 1966 a)

A microheterogeneity of the fibrinopeptides has been observed in some species Two analogues of human fibrinopeptide A have been isolated fibrinopeptide Y —which is fibrinopeptide A less the N terminal alanine—and fibrinopeptide AP —which is fibrinopeptide A with a phosphorylated serine (Blomback *et al* 1966 b) *Canine fibrinopeptide A is at least partially phosphorylated* (Osbahr *et al* 1964 a Osbahr 1966) and one or two tyrosine residues in canine fibrinopeptide B occur as tyrosine O sulphate (Krajewski and Blomback 1968) Fibrinopeptides B lacking the C terminal arginine have in addition been isolated from fibrinogen of some species (Pirkle and Blomback 1966 Blomback 1967) These authors presumed that the desargınino fibrinopeptides were produced by enzymatic de *gradation during the procedure of isolation This assumption was supported by*

the finding that fibrinopeptide B from spontaneously coagulated elk blood completely lacked the C terminal arginine (Blombäck 1967) and by the preliminary work of Teger Nilsson and Blombäck (1967) on degradation of the fibrinopeptides

An interesting physiological effect of the fibrinopeptides was described by Gladner and co workers Human fibrinopeptide A and bovine fibrinopeptide B potentiated the activity of bradykinin on certain smooth muscles (Gladner *et al* 1963 Osbahr *et al* 1964 b) This also seemed to apply to canine fibrinopeptide A (Gladner 1966) Colman *et al* (1967) observed a vasoconstrictor effect of bovine fibrinopeptide B Recently Bayley and co workers (1967) claimed an effect of fibrinopeptides on the pulmonary circulation of some experimental animals Copley *et al* (1967) reported the fibrinopeptides to have an effect on the blood capillary wall

Present investigation

The indirect action of the fibrinopeptides on smooth muscles indicates that these peptides may have a physiological role in regulation of the blood circulation An investigation of the fate of the fibrinopeptides during intravascular coagulation was therefore considered of interest It was also believed that knowledge about the fate of fibrinopeptides would be of value in particular for the diagnosis of intravascular coagulation Release of the fibrinopeptides from fibrinogen has been demonstrated in experiments with purified components *in vitro* and it is generally assumed that the same mechanisms operate during fibrin formation *in vivo* This has not, however been proved and little is known about the degradation catabolism and excretion of the fibrinopeptides after their release from fibrinogen during intravascular coagulation The finding of small amounts of desarginino fibrinopeptides in clot supernatants and the presence of carboxypeptidases in blood (Erdos *et al* 1964) indicated that released fibrinopeptides might be degraded in the blood

The present investigation deals with the degradation of the fibrinopeptides in blood *in vitro* and *in vivo*

In vitro degradation of fibrinopeptides in serum

Purified human and canine fibrinopeptides were incubated in small volumes of homologous serum (VI and VII) After incubation the protein in the samples was precipitated with trichloroacetic acid and the protein free supernatant was submitted to high voltage electrophoresis on paper after removal of the acid Several fractions appeared during incubation indicating a degradation of the fibrinopeptides The electrophoretic fractions were eluted from the papers and

their amino acid composition was determined after hydrolysis The following conclusions were drawn on the basis of the amino acid analyses and the electrophoretic mobilities

Human fibrinopeptide A seemed to be degraded from both the C terminal and N terminal ends (VI) The C terminal arginine was split off but no further degradation from the C terminal end was observed Both the N terminal alanine the penultimate aspartic acid and probably also small amounts of the next amino acids serine and glycine were released from the N terminal end

Human fibrinopeptide B was degraded from its C terminal end by the release of arginine (VI) Fibrinopeptide B was not regraded from its N terminal end which was to be expected since a pyroglutamic acid occupies this position

Canine fibrinopeptide A was also degraded in canine serum (VII) The C terminal arginine was released but no degradation from the N terminal end was observed

As far as *canine fibrinopeptide B* is concerned there were some indications that C terminal arginine and N terminal histidine were split off (Miller and Teger Nilsson 1968) The yield of the degraded peptide B fragments was however very low

To obtain information on the rate of arginine release human fibrinopeptides were incubated in homologous serum for varying periods The arginine released was then separated from the fibrinopeptides by means of ion exchange chromatography on small columns The amount of free arginine was determined by a modification of the Sakaguchi reaction (VI)

Arginine was released at a slow rate from fibrinopeptide A and only to about 30 per cent The release of arginine from fibrinopeptide B was rapid and complete in about 30 minutes Thus degraded human fibrinopeptide B may appear rapidly *in vivo* The results support the earlier view that fibrinopeptide B without arginine isolated from several species is due to enzymatic degradation

The study of the release of arginine gave as a by product some serum containing fibrinopeptides and degraded fibrinopeptides in high concentrations An attempt was made to isolate these peptides (VI) The serum proteins were precipitated with trichloroacetic acid The supernatant was collected and the remaining trichloroacetic acid extracted with ether After evaporation and lyophilization the residues were desalted on a column of Sephadex G 10 The fractions in the first ninhydrin positive peak were collected and applied to a column of Dowex 50 which was eluted by stepwise increase in pH The chromatogram of the samples containing fibrinopeptide A showed seven ninhydrin positive peaks whereas the chromatogram of the samples containing fibrinopeptide B had only two main peaks The fractions of each peak were collected lyophilized and submitted to electrophoresis Those fractions which showed only one band in electrophoresis were directly analyzed The other fractions were further separated by means of

preparative electrophoresis on paper and then analyzed for their amino acid composition The amino acid composition and electrophoretic mobility of the frac tions from the fibrinopeptide A containing samples were in agreement with un changed fibrinopeptide A

NH_2 Ala Asp Ser Gly Glu Gly Asp Phe Leu Ala Glu Gly Gly Gly Val Arg OH

and with the following derivatives

NH Asp Ser Gly Glu Gly Asp Phe Leu Ala Glu Gly Gly Gly Val Arg OH
NH_2 Ser Gly Glu Gly Asp Phe Leu Ala Glu Gly Gly Gly Val Arg OH
NH_2 Ala Asp Ser Gly Glu Gly Asp Phe Leu Ala Glu Gly Gly Gly Val OH
NH Asp Ser Gly Glu Gly Asp Phe Leu Ala Glu Gly Gly Gly Val OH

The peptides isolated from the samples containing fibrinopeptide B were in agreement with the following structures

Glu Gly Val Asp Asp Asp Glu Glu Gly Phe Phe Ser Ala Arg OH
Glu Gly Val Asp-Asp Asp Glu Glu Gly Phe Phe Ser Ala OH

i e, unchanged fibrinopeptide B and desarginino fibrinopeptide B

These results fully confirm those obtained by the small scale experiment with electrophoretic separation alone and show that it is possible to isolate fibrino peptides and degraded fibrinopeptides from serum The concentrations of the fibrinopeptides in the starting material of this experiment were however much higher than those which can be expected to occur in blood during intravascular coagulation

Isolation of the fibrinopeptides from blood of thrombin treated dogs

Intravascular coagulation was induced in dogs by slow intravenous infusion of thrombin (VII) When the dogs blood had become incoagulable with throm bin the animals were exsanguinated Plasma was immediately separated and the proteins precipitated The material in the supernatant was collected at pH 2 8 on a column of Dowex 50 from which it was eluted at pH 6 8 The eluate was concentrated and then desalted on a column of Sephadex G 10 The desalted peptide material was further fractionated on ion exchange resin by elution with a pH gradient. The eluate was analyzed with the ninhydrin reaction before and after alkaline hydrolysis and divided into 13 fractions which were analyzed by thin layer electrophoresis at pH 5 5 A large number of spots were observed i the different fractions At this pH the fibrinopeptides and their expected derivatives move to the anode Only those spots which appeared in the anode region were therefore taken into consideration for further studies Altogether

13 acidic fractions were isolated from the thin layer plates and analyzed for amino acid composition Only two of these fractions had a composition which were compatible with that of fibrinopeptides The amino acid analysis of one of the fractions were in reasonably good agreement with the structure given by Blomback *et al* (1965 c) for canine fibrinopeptide A The amino acid composition of the other fraction was similar to desarginino fibrinopeptide A The electrophoretic mobilities of the fractions at pH 5 5 were also comparable to those of canine fibrinopeptide A and degraded fibrinopeptide A

To obtain further evidence that the isolated fractions contained mainly fibrinopeptide A and fibrinopeptide A less arginine the fractions were subjected to tryptic digestion and the peptide map after electrophoresis compared with a tryptic digest of authentic canine fibrinopeptide A Trypsin is expected to yield two pieces by hydrolysis of a lysyl glutamic acid bond Three ninhydrin positive spots were observed one of which was unchanged fibrinopeptide The peptide maps of the analyzed fractions as compared to that of the reference supported the view that the unknown fractions from the blood consist mainly of fibrinopeptide A and fibrinopeptide A less C terminal arginine Thus the same peptide derivative was obtained during incubation in serum *in vitro* and during intravascular coagulation

Only small amounts of the fibrinopeptides were however found The low yield may have several explanations Fibrinopeptide A may not have been quantitatively released from fibrinogen *in vivo* and more extensive degradation may have occurred *in vivo* than *in vitro* Released fibrinopeptide may have been rapidly excreted in the urine which was not collected in the present investigation Peptide material may also have been lost during the purification procedure

No fibrinopeptide B or derivatives of fibrinopeptide B were found in the blood of the thrombin treated dogs This might indicate that fibrinopeptide B is not released from fibrinogen during coagulation *in vivo* It is also possible that released fibrinopeptide B had been degraded *in vivo* excreted in the urine or lost during the purification procedure

Aspects of fibrinopeptide degradation

The present study shows that the fibrinopeptides are degraded by exopeptidases in the blood The degradation of human fibrinopeptide A from its N terminal end is of special interest in view of the fact that a peptide—denoted as fibrinopeptide Y and shown to be fibrinopeptide A less N terminal alanine—has been isolated from purified fibrinogen (Blomback *et al* 1966 b) The α(A) chain of the fibrinogen molecule has in addition to alanine small amounts of the amino acid penultimate to alanine *i e* aspartic acid as N terminal residue (von Korff *et al* 1963) Although this microheterogeneity of fibrinogen might be attributable

to different genetically determined variants the present investigation suggests that it is more likely due to enzymatic degradation of fibrinogen in blood The prospect of a plasma protein like fibrinogen being degraded in the blood raises the question whether other proteins can be degraded in a similar fashion

Fibrinopeptides played no role in the peripheral vasodilating activity of thrombin (IV and V) Fibrinopeptides may however have other circulatory effects e g on the pulmonary circulation as suggested by Bayley et al (1967) The physiological effects of human fibrinopeptide A have been related to the N terminal sequence namely Ala Asp-Ser Gly Glu (Gladner 1966) In a discussions of the physiological effects of human fibrinopeptide A in man it must be taken into consideration that this sequence might be destroyed when fibrinopeptide A is circulating in the blood

Intravascular coagulation is postulated to occur in a number of diseases including such diverse disorders as premature separation of the placenta septicaemias and intravasal haemolysis The symptoms of disseminated intravascular coagulation with secondary fibrinolysis and those of primary fibrinolysis are similar and a differentiation between these two conditions is almost impossible at present A differentiation is however of importance since the treatment is not the same A strong evidence of the occurence of intravascular coagulation would be the finding of fibrinopeptides or of fibrinopeptide derivatives in blood or urine

Summary

Purified human and canine fibrinopeptides were incubated in homologous serum The products were separated by means of electrophoretic and chromatographic methods and identified by amino acid analyses Arginine was split off from the C terminal end of human fibrinopeptide A Alanine aspartic acid and possibly also serine and glycine were released from its N terminal end C terminal arginine alone was split off from human fibrinopeptide B and canine fibrinopeptide A Arginine was released slowly from human fibrinopeptide A and only to about 30 per cent The of release of arginine from human fibrinopeptide B was fast and complete

Fibrinopeptides were isolated from the blood of thrombin treated dogs by means of protein precipitation gel filtration, chromatographic and electrophoretic methods Two fractions which could be related to the fibrinopeptides were found Electrophoretic mobility of the fractions their tryptic peptides and their amino acid composition indicated that the fractions consisted of fibrinopeptide A and fibrinopeptide A less C terminal arginine

GENERAL SUMMARY

A procoagulant from human postpartum serum was purified and characterized Its properties in coagulation assays some of its chemical properties and its biological effects suggest that the procoagulant is a tissue thromboplastin Since no such procoagulant was found in normal serum it is probably derived from the placenta

The effects of tissue thromboplastin and thrombin on haemodynamics in dogs were studied Infusion of purified or dialyzed tissue thromboplastin into a femoral artery had no local peripheral vasodilating effect Similar infusions of small amounts of thrombin did however give rise to local vasodilatation Intravenous infusion of large amounts of thrombin in defibrinogenated animals produced hypotension Fibrinopeptides or plasma kinins played no role whereas the presence of blood cells was of importance for the vasodilating activity of thrombin

Degradation of fibrinopeptides was studied *in vitro* and during intravascular coagulation C terminal arginine was released from human fibrinopeptides A and B and from canine fibrinopeptide A In addition a stepwise degradation took place from the N terminal end of human fibrinopeptide A during incubation in homologous serum Two peptides probably fibrinopeptide A and fibrinopeptide A less C terminal arginine were isolated from the blood of dogs after thrombin induced intravascular coagulation

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